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STEAM PRETREATMENT OF WOOD IN RELATION TO ENZYMATIC HYDROLYSIS

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FINAL REPORT

JANUARY, 1988

DSS FILE NO. 05SR.31926-4-5022

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ABSTRACT

The effects of variables in steam pretreatment cf aspenwood (temperature, time, moisture content and SO_2 catalysis) have been determined, with and without explosion, on the resulting composition and enzymatic hydrolysis. Combination treatments (steaming + wet-milling, or steaming + H_2O_2 treatment) further increase accessibility. Results show that accessibility is not increased by explosion, but is increased by hydrolysis of both xylan and lignin, and by H_2O_2 oxidation of lignin. Accessibility correlates well with content of residual alkali-insoluble lignin (the most important factor), but not with total residual lignin, and not with xylan. Free alkali-soluble lignin has no effect. H_2O_2 increases accessibility by reducing the content of alkali-insoluble lignin, which presumably restricts cell-wall swelling.

SO_2 catalysis increases hydrolysis relative to undesirable pyrolysis and xylose destruction. Variables (ratio of wood/gun volume, SO_2/wood, temperature, time and moisture content) have been studied, and further improvements by H_2O_2 measured. Analytical methods for substrates and enzymatic hydrolysates are discussed, and the reason for discrepancies shown. Improved HPLC methods for enzyme assay (FPU and β-glucosidase) are reported. Crystallinity is not decreased by steam treatment, with or without explosion.

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ABBREVIATIONS USED

SEA	steam-exploded aspenwood
SHA	steam-heated aspenwood (without explosion)
WS	water-soluble fraction
WI	water-insoluble fraction
WIA	water-and-dilute-alkali-insoluble fraction

Combinations of the above, e.g.

SEA-WS	SEA-WI
SHA-WIA	SHA-WIA

Abbreviation preceded by SO_2- indicates SO_2-catalyzed.

Abbreviation followed by $-H_2O_2$ or $/H_2O_2$ indicates H_2O_2 treatment.

e.g. SO_2-SHA-WIA/H_2O_2 means SO_2-catalyzed steam treatment (without explosion) followed by water washing, then by dilute alkali (0.1N) washing and treatment with H_2O_2.

OBJECTIVES (AT BEGINNING OF CONTRACT PERIOD)

1) To confirm, if necessary, the tentative conclusion that the significant pyrolysis occurring during steam explosion process does not contribute significantly to the enzyme digestibility of wood and is therefore an undesirable side reaction.

2) To determine conditions which minimize pyrolysis (if pyrolysis is found in (1) to be an undesirable side reaction), and which maximize the desirable process of hydrolysis, by acid impregnation of the chips, and to examine the effect on: (a) byproduct lignin, (b) separation of components, (c) inhibitor formation and (d) the state of hemicellulose

3) To confirm, if possible, that small-size air-dry chips can be used instead of green wood, to reduce substantially high-pressure steam requirements, and to determine the extent of any resulting changes in inhibitor formation, completeness of separation of components, and enzymatic hydrolyzability

4) To examine as in (3) the possibility of using air-dry chips after acid impregnation

5) To compare the savings of high pressure steam resulting from the use of air-dry chips, with the added cost of predrying the chips

6) To examine the effect, if any, on the quality of by-product lignin, resulting from a pre-heating of the charge of wood chips before the loading of these chips into the gun to reduce further the consumption of high-pressure steam

7) To attempt to adapt the steam-explosion process to softwoods such as spruce, through the use of dilute sulfuric acid or other catalysts, and, if successful in obtaining adequate enzymatic hydrolysis, to examine the molecular weight, solubility and degree of condensation of the lignin

8) To determine the feasibility of withdrawing steam from the gun (for re-use in a second gun), before explosion from a much lower pressure and to determine the quality of the resulting exploded wood

9) To combine the best approaches or conditions found under each of the above objectives and to determine whether or not many of them can be used simultaneously without adverse effect, and to modify the two-litre steam gun at Forintek in order to test experimentally the re-using of high-pressure steam withdrawn from the gun under these conditions

10) To determine what necessary physical changes are caused in the structure of wood (e.g. in the S1, S2 or S3 layers) by steam explosion, in order that enzymes can gain access

11) To correlate structural changes observed by EM during steam-explosion treatments of increasing severity, with the development of increased accessibility to enzyme systems

12) To correlate, if possible, increasing accessibility with the development of pores sufficiently wide to accommodate molecules of enzyme, as measured by solute exclusion, and with modification of the pore surface by extraction or mild oxidation, and to confirm reports that the crystallinity of the cellulose is not significantly changed from that of the

original wood.

The above objectives, which are reviewed below in the Introduction Section, fall generally under the following areas:

- Pyrolysis (Objectives 1 & 2)
- Steam Consumption (Objectives 3,4 & 5 - air-dry,
  Objective 6 - preheating,
  Objective 8 - reuse)
- Softwood Utilization (Objective 7)
- Combination of Best Approaches (Objective 9)
- Physical Changes (Objectives 10, 11, 12)

OBJECTIVES (FOR 3RD YEAR OF CONTRACT)

After the first two years of the contract, the objectives still not completed were modified somewhat in the light of the findings, to the objectives listed below.

1) SO_2 Catalysis (related to old Objective 7)

   The effect of pre-impregnation of aspenwood and sprucewood chips with SO_2 will be compared with the effect of introducing gaseous SO_2 into the chips simultaneously with the steam, or after the steam, on the extent of the catalysis produced by the SO_2. Any differences, resulting from differing rates of temperature rise, will be measured. The effect of the principal variables (steam temperature, treatment time, and SO_2 loading) will be determined for sprucewood.

2) Combination SO_2-H_2O_2 (related to old Objective 9)

   The possibility, that a hydrogen peroxide treatment of the

water-washed and alkali-washed product of SO_2-catalyzed steam treatment of aspenwood and sprucewood can produce a significant improvement in enzymatic hydrolysis, will be examined. The enzyme levels to be used will be low enough to leave room for improvement by the peroxide. If an improvement is obtained, the conditions of the peroxide treatment will be varied to optimize the benefit.

3) Sapwood - Heartwood Decayed (a new objective)

Samples of sapwood and of heartwood will be separated from bolts of sound aspenwood and sprucewood, and will be chipped. Samples of partially decayed aspenwood will also be chipped. Steam treatments will be done and any difference in the response to the treatment ascribable to differences in sapwood, heartwood or decayed wood, will be observed by chemical analysis and by enzymatic hydrolysis.

4) Factors affecting accessibility (related to old Objectives 10, 11, 12)

Samples of aspenwood will be steam treated with steam at 240°C for 80s (i.e. for long enough to solubilize most of the pentosan), and for a longer time such as 160 or 200 s (i.e. long enough to improve further the enzymatic hydrolysis). After water-washing to remove solubilized pentosan, enzymatic hydrolysis will be performed, both before and after alkali extraction (to remove solubilized lignin). If, as expected, it is confirmed that the longer treatment gives a better enzymatic hydrolysis, these substrates will then be characterized as thoroughly as is feasible in an attempt to determine which characteristics

were principally responsible for the differences in enzymatic hydrolysis. If possible, the characteristics measured will include: content of pentosan, lignin and cellulose, crystallinity index, degree of polymerization of the cellulose, degree of swelling, and possibly pore-size distribution.

If possible the experiment will be repeated with another pair of samples (e.g. steam treated wood, before and after peroxide treatment which should alter the chemical composition only slightly but should enhance enzymatic hydrolysis. Again the characteristics will be measured, for the purpose of determining which factor caused the improvement.

5) Substrate Characterization (a new objective)

An attempt will be made to determine the cause of the discrepancy between the HPLC and anthrone determinations of the content of cellulose or other glucan. Samples of pure glucose, and of a relatively pure microcrystalline cellulose, will be hydrolyzed by the Klason treatment and will be analyzed by both the HPLC and anthrone methods. If no discrepancy is found, the possibility will be examined of interference with the anthrone method, by lignin or by break-down products (other than furfural) present in steam-treated wood.

6) Air-Dry H_2SO_4-Impregnated (old Objective 4)

Aspenwood, impregnated with dilute sulfuric acid, will be air-dried and steam-treated. The steam temperature will be lowered until either furfural formation becomes negligible,

or pentosan solubilization and enzymatic hydrolysis become inadequate, in order to determine whether or not air-drying could be used.

7) Preheat Chips (similar to old Objectives 5 and 6)
Green aspenwood chips will be preheated to approximately 100°C before loading into the steam gun for steam treatment. Any adverse effect of the preheating will be observed by analysis and enzymatic hydrolysis.

8) Steam Re-Use (old Objective 8)
A second unused 2-litre barrel of an old steam gun will be closed at one end, by a metal plate, and at the other end by the Parr bomb lid. It will be connected to the existing 2-litre steam gun and to the steam boiler. Aspenwood chips will be steam treated in one gun, and the steam then bled off into a fresh charge of chips in the second gun until equilibrium is approximately reached. Fresh steam from the boiler will then be used to top off the second gun and complete the cook. Any adverse effect of the steam re-use will be observed on enzymatic hydrolysis and fermentation. Depending on the outcome of Objective 1, the re-use of SO_2 will also be attempted.

INTRODUCTION

Pyrolysis

The occurrence of pyrolysis and other degradative reactions during steam pretreatment of wood has been well established. Forintek (1,2) reported gaseous pyrolysis products in the steam

gun after treatment of aspenwood with saturated steam at 240°C. After 2 minutes at this temperature, the content of carbon dioxide in this gas amounted to 2% of the original dry weight of the wood. Weight losses of up to about 16%, and approximately similar losses in pentosan content have been observed (1,3). Also, a pronounced decrease in the oxygen content was revealed by elemental analysis (4) of the non-volatile residual material. This decrease reflected the loss of relatively oxygen-rich, gaseous or volatile products, such as carbon dioxide and water, which occurred to a sufficient degree to more than balance the gain in oxygen resulting from the accompanying process of hydrolysis.

Hydrolysis, particularly of the hemicellulose, has been regarded as a desirable reaction during steam treatment, because it solubilizes much of the hemicellulose and renders the cellulose accessible to the cellulase enzyme system (5). Whether or not pyrolysis and other degradative reactions serve any useful purpose, is not clear. Forintek (1) treated thin aspenwood wafers in a stream of dry (superheated) steam at atmospheric pressure for times ranging from 4 to 20 minutes. Although this treatment destroyed from 32 to 72% of the pentosan, it did not solubilize any significant amount of the surviving pentosan. Furthermore, these partially pyrolyzed products were not good substrates for enzymatic hydrolysis, even after washing with water and with dilute alkali. Pyrolysis, at least under these dry conditions, was concluded to be undesirable. The possibility remained, however, that pyrolysis or other degradation reactions,

under wet conditions, either following or during partial hydrolysis in saturated steam, might have some beneficial effect. Such an effect might conceivably result from a melting and increased flowing of the pyrolyzed product, and a resulting exposure of more of the cellulose.

Even assuming pyrolysis to be undesirable, the best way to minimize it is not clear. In the manufacture of dissolving-grade pulps by the kraft process, a water or dilute mineral acid prehydrolysis is employed to solubilize the hemicelluloses of the wood before pulping. Springer and Harris (6) have shown that the use of dilute mineral acid (e.g. 0.4% sulfuric at 170°C) results in a more selective removal of xylan from aspenwood, with less loss of xylose, or of xylose oligomers, by degradation. They ascribed this finding to the greater stability of sugars in acid solution, maximum stability being in the pH range from 2.5 to 3.5. They considered xylose degradation, even at pH 4, to be hydroxyl-ion catalyzed. Forintek (1) has also shown that impregnation with 0.2% sulfuric acid similarly greatly reduces the loss of xylose and of xylose oligomers during steam treatment at 240°C. Acid impregnation also results in a better substrate for enzymatic hydrolysis. There is, however, a reluctance in Europe, particularly in Germany, to use mineral acids or sulfur dioxide as catalysts, primarily because of environmental considerations (7). In addition, the requirement for more costly acid-resistant equipment, and the complexity resulting from acid impregnation, neutralization and handling of calcium sulfate downstream from the neutralization, detract from the advantages

of using acid.

It is also not clear from the literature, whether high temperatures and short times of treatment, or lower temperatures and longer times, are preferable. Some of the literature on the Masonite Process (8), and on the steam explosion (9,10), stresses the importance of high temperature (approx. 250°C) to soften the wood components. The explosion can then disrupt the softened wood structure. Forintek (1,2), however, has shown the explosion to be unnecessary for subsequent enzymatic hydrolysis.

Puls and co-workers (11) recently reported increased extraction of hemicelluloses, with increasing temperature of steam treatment from 170° to 210°C. Raising the temperature from 200° to 210°C, however, also resulted in a sharp drop in the combined amount of xylose and xylose oligomers in the water-soluble fraction, as a result of degradation. Formation of fermentation inhibitors also increased at 210°C. The yield of glucose obtained on enzymatic hydrolysis, however, increased with temperature of pretreatment, and was greatest at the highest temperature studied (i.e. 210°C), which is consistent with the greatest removal of hemicellulose. The glucose yield at 210°C was actually one-third greater than that at 200°C. Puls concluded that, "the extraction of hemicellulose was most efficient at 200°C, above which temperature part of the sugars was destroyed", and that "at 210°C the overall carbohydrate yield decreased sharply".

It is important to note, however, that all of these treatments were for the same steaming time of 10 minutes. By that time, the

amount of xylose monomer in the wood steamed at 210°C was approximately double that at 200°C. It is not surprising, therefore, that greater degradation of xylose occurred at 210°C. A more meaningful comparison, at the different temperatures, would have been of the recovery of xylose and xylose oligomers, in the extract, after treatment times selected to give some constant and reasonable glucose yield on enzymatic hydrolysis. It is the rate of development of cellulose accessibility relative to that of carbohydrate destruction, that is important.

An earlier and more elaborate comparison (12), which was reviewed by Forintek (1, Appendix 1), showed that at the three temperatures of 259, 250 and 226°C there was no significant difference between the maximum yields of glucose obtained by enzymatic hydrolysis, after the optimum times of steam treatment at each temperature. The corresponding yields of xylose, obtained after these same optimum times for glucose, decreased with decreasing steam temperature. These data suggest that for equally high glucose yields at these three steam temperatures, the conservation of xylan increased with the temperature and was highest at the highest temperature studied (i.e. 259°C). Unfortunately, the scatter of these data, particularly that of the xylose yields, was so great as to render this apparent trend of questionable significance. It is not clear, therefore, whether the activation energy of hemicellulose hydrolysis is higher or lower than that of the destruction processes. If the hemicellulose destruction occurs predominantly through the free monomeric sugars after hydrolysis, then the situation might be

analogous to that during dilute acid hydrolysis, where the highest sugar yields are obtained after short treatment times at the highest temperatures (13).

Steam Conservation

It has been shown (1) that the theoretical weight of high pressure steam at 240°C, required to heat green aspenwood to steam temperature, and to pressurize the apparatus, is approximately 90% of the dry equivalent weight of the wood. The theoretical steam requirement would be more than cut in half if air-dry rather than green wood were used, because more than half of the heat required is consumed in heating the moisture initially present in green wood. Whether or not the possible saving in high-pressure steam would have a worth-while effect on the over-all economics of the pretreatment, depends, in part, on the cost assumed for the high-pressure steam, and on the cost associated with the drying. Some early assessments rated steam cost as second only to that of the wood itself. In other assessments, where no market was assumed for the by-product lignin, and this lignin was assumed to be burned for its calorific content, little value was assigned to the steam. In all cases, capital cost of the equipment contributed the greatest amount to the cost of the final products.

An early study (12), reviewed by Forintek (1, Appendix 1), indicated that the optimum steaming time decreased with decreasing initial moisture content. Maximum yields of both glucose and xylose, at their respective optimum steaming times,

appeared to decrease (although erratically) with decreasing moisture content below 35 or 50% (moisture-containing basis). Forintek, however, showed that, when thin air-dry aspenwood wafers were treated with steam at 240°C, virtually identical results were obtained as with small green chips (4.7 mm thick, in the fiber direction). Accordingly, virtually identical curves of pentosan present in the aqueous extracts, and of pentosan remaining in the water-insoluble fractions, were obtained, when plotted against steaming time.

Another comparison has been reported by Forintek for more carefully matched air-dry and green wood from the same batch of small chips. In this case, only very slightly lower yields of glucose and of total reducing sugars were obtained from the air-dry wood after steam explosion, water washing and enzymatic hydrolysis. These lower yields, however were paralleled during the steam treatment by slightly less pentosan destruction and by slightly less pentosan solubilization, implying a slightly less-complete cook, in the case of the initially air-dry wood. These results showed that essentially identical results could be obtained from the steam treatment of air-dry and green aspenwood, at least when a small particle size was used. At the most, only a slight adjustment in steaming time might be necessary. The scatter observed in the earlier study may possibly have resulted from the larger chip size and from the difficulty in obtaining a uniform moisture content in chips partially air-dried on the floor.

In principle, the consumption of high pressure steam could be reduced substantially by preheating the chips. This preheating could be done outside of the pressure vessel before loading (perhaps as the final stage of a pre-drying operation), or inside the vessel by lower pressure steam. Because the enthalpy per kg of high-pressure saturated steam at 240°C is only 5% higher than that of saturated steam at as low a temperature as 100°C, very little additional gain in moisture content would result from preheating with low pressure steam. Steam, recovered from bleed-down of high pressure steam from a completed 240°C treatment in a second pressure vessel, might also be used. The effect of such measures, on subsequent enzymatic hydrolysis and fermentation, and on the properties of the lignin, have not apparently been reported.

Softwood Utilization

Softwoods are generally recognized as much more refractory than hardwoods, to the development of cellulose accessibility by steam treatment (14). Treatment, however, is substantially improved by impregnation with dilute mineral acid. Grethlein (5) has reported that the initial rate of enzymatic hydrolysis of both hard and softwoods, pretreated by mild acid hydrolysis, is linearly correlated with the pore volume of the substrate accessible to a nominal diameter of 51 Å representative of the size of the cellulase. The fact that pinewood, after a comparable pretreatment, gave lower yields of glucose than did hardwoods, was explained by Grethlein as due to the smaller increase in the required size range of pore volume. He suggested

that the hemicellulose in the lignocellulose of pinewood is dispersed in smaller pores than is that of hardwoods. Partial hydrolysis and removal of this hemicellulose accordingly opens-up smaller pores in the pinewood. The possible adverse effect on the quality of the softwood lignin, resulting from the more drastic pretreatment required to obtain adequate pore volume, has apparently not been investigated.

Air-Dry, H_2SO_4-Impregnated Wood

During the early stages of steam treatment of wood which has been impregnated with dilute sulfuric acid, much of the dilute acid is driven out of the wood by expansion of trapped air or gases of decomposition. This part of the impregnating acid runs down to the bottom of the apparatus and is ineffective in the hydrolysis. Since air-dry aspenwood has been shown to steam treat to give a good substrate for enzymatic hydrolysis, it is possible that the air-drying of acid-impregnated wood would do likewise. Furthermore, loss of acid by out gassing would be minimized; the higher acid concentration resulting from air-drying would permit the use of a lower temperature, and the requirement for high-pressure steam would be reduced. The dehydration of the more concentrated xylose to furfural, in the more concentrated sulfuric acid, would have to be avoided.

Physical Changes

The development of micropores in the cell walls, large enough to accommodate molecules of cellulase enzymes of effective diameter 40-50 $\mathring{A}$, is probably not the only requirement for the development of accessibility. Ladish and co-workers (15), for example, have

recently reported that micropore changes (in the 40-160 A range), caused by pretreatment of cornstalk residue with 1.5 N NaOH or with 1.5 N iron sodium tartrate, do not explain the enhanced fermentation results obtained with rumen fluid. They concluded that external or macropore surface properties are a key factor in utilization of corn residue by cellulolytic bacteria. Membrane-bound cellulase enzymes, as well as adsorbed cellulases, are known to be responsible for fiber digestion by rumen microbes. The bacteria themselves (0.3-2.0 um by 1.0-6.0 um) are, of course, much too large to enter the micropore structure of the cell walls, whereas the bacterial cellulases were reported to have effective diameters of 40-160Å (i.e. 4-16 nm).

David and co-workers (16) reported that, pretreatment of Eucalyptus wood meal with dilute acid reflux at 100°C, removed 68% of the hemicellulose, but gave only a slight increase in the yield of glucose (from 0% to 2%), on subsequent enzymatic hydrolysis with the cellulases of Trichoderma viride. In marked contrast, pretreatment at room temperature with sodium hypochlorite, acidified to between pH 7 and 9, gave a large increase in yield of glucose. The authors ascribed this increase to the elimination of an important fraction of the lignin. Part of the oxidized lignin was solubilized, while another part remained trapped in the cell-wall structure.

In an earlier study (17), Saddler and co-workers showed that, with increasing time of steam treatment of aspenwood at 250°C, increasingly large amounts of pentosan were solubilized (in

water), and increasingly large yields of glucose were obtained on subsequent enzymatic hydrolysis. Approximately 12% of the lignin was rendered water-soluble after each of the steaming times. Much of the remaining (water-insoluble) lignin, although it was still held in the wood structure, was extractable by dilute alkali (0.1N), or by organic solvents, in amounts which also increased with increasing time of steaming. Removal of this alkali-soluble lignin resulted, at best, in only a small increase in the yield of glucose on enzymatic hydrolysis. A mild oxidative treatment of the alkali-washed steam-treated aspenwood, however, with only 2% of its weight of sodium chlorite, resulted in a further significant increase in the subsequent yield of glucose. This increase occurred even though the chlorite treatment had reduced the lignin content by only 1.6%, and the pentosan content was unchanged. Surface modification, rather than pore development was therefore suggested.

The possibility that oxidative modification of the cellulose increased the enzymatic attack, was considered by David and co-workers. They reported that, in the absence of lignin, the reaction of pure cellulose with partly acidified sodium hypochlorite results in the formation of large amounts of aldehyde, ketone and acid groups. These groups, however, decreased the reactivity of the cellulose toward enzymatic hydrolysis.

Oxidative pretreatment of lignocellulosic materials at 25°C with hydrogen peroxide at pH 11.5, has been described by Gould (18,19). Although most effective when applied to

monocotyledonous plants, this pretreatment was also effective with oakwood. For example, the efficiency of the conversion of cellulose to glucose, by the cellulase of Trichoderma reesei, was increased from 22% to 53% for oak shavings, and from 27% to 93% for wheat straw, after only 6 hours of peroxide pretreatment. This pretreatment resulted in partial delignification but, at this pH of 11.5, most of the hemicellulose was retained in the insoluble cellulosic fraction. Water-soluble lignin degradation products (mostly carboxylic acids) were reported to be non-toxic to microbial growth, and peroxide pretreatment was suggested for increasing the digestibility of lignocellulose for use as ruminant fodder.

In some of the patent literature on the steam explosion process (9,10), the development of cellulose accessibility is ascribed almost entirely to physical changes occurring during the explosion and, in particular, during extrusion of the hot lignocellulose through a die in the outlet from the pressure vessel. The lignocellulosic material reportedly issued from the extrusion die, in particulate form, with the lignin rendered into particles substantially in the range from 1 to 10 um. The importance of operating at temperatures above the softening points of the wood components was stressed. Marchessault and St. Pierre (20) also believed that the physical effect of explosive fracture, in this process, contributed to the profound increase in enzyme accessibility. Other workers (8,21,22), however, have recognized that the process is largely chemical in nature.

SO_2 Catalysis

It has become apparent that one of the most costly steps, in the enzymatic hydrolysis of wood, is enzyme production. The most cost-effective improvement in pretreatment, would be in the production of a more accessible substrate which would hydrolyze faster and more completely while requiring as little enzyme as possible. Cellulose accessibility increases with increased time of steaming. This improvement continues to beyond the time where much, or most, of the xylan was destroyed. Added acidic catalysts can conserve the xylose, and permit lower steam temperatures to be used. Among these catalysts, sulfur dioxide is probably the most practical. It is cheap, less corrosive than sulfuric acid, easier to use, and appears to be as good as sulfuric acid. Its use, however, results in the incorporation of a small amount of sulfur, apparently in the lignin fraction. For some uses of the by-product lignin, this sulfur may be undesirable. It is probably present as sulfonic acid groups, which, being strongly acidic, may play a role in the catalysis.

Conflicting reports have indicated, 1) that the SO_2 can be introduced with the steam into the Stake Reactor (Wayman), and, conversely, 2) that the SO_2 cannot catalyze the steam treatment satisfactorily when introduced with the steam into the top of a Masonite-type steam gun (Mackie, and private communications). At Forintek, only pre-impregnation with SO_2 has been used. The solubility of SO_2 in water decreases with increasing temperature and is practically zero at steam-gun temperatures of about 200°C. Although the solubility should be greater at higher pressures (as

is the case, for example, of carbon dioxide in soda water), it is the partial pressure of SO_2 that matters, and not the very much higher partial pressure of the steam. Under the conditions used, the partial pressure of SO_2 would be unlikely to exceed even one atmosphere.

In sulfite pulping there is a base present in the water, to retain part of the SO_2 in salt form. In the steam gun, however, it would be expected that SO_2 would be driven off as a result of the rapidly rising temperature. It is not clear, therefore, whether sulfonation occurs during warm-up, before the SO_2 is driven out of the pre-impregnated moist chips, and the resulting non-volatile sulfonic acid groups catalyze the hydrolysis; or whether there is an equilibrium established between a very little dissolved SO_2 and a much larger amount of SO_2 in the vapor state.

It is important to understand what actually occurs, because of its bearing on the most desirable heating schedule, the method of introducing the SO_2, the possibility of recovering and re-using SO_2 in the vented steam, the uniformity of the product, the sulfur content of the byproduct lignin, and environmental considerations.

Combination SO_2-H_2O_2

The accessibility of aspenwood, after steam treatment without added catalysts, and after extraction with water and alkali to remove the hemicellulose and lignin streams, was shown during the first year of the contract to be significantly increased by a peroxide secondary treatment. It is not clear exactly how the

improvement was achieved. It is unlikely that the small additional lignin removal is the whole story. Not only is the enzymatic hydrolysis faster and more complete, as a result of the peroxide treatment, but the enzyme loading can be reduced as well.

It is possible that a secondary peroxide treatment would also further improve the already good substrate produced by steam treatment when an SO_2 catalyst was used. (Objective 2). Furthermore, additional improvement could conceivably result from changes in the secondary treatment conditions, such as increasing the temperature to reduce the treatment time, bearing in mind that there is no advantage in conserving the DP of the cellulose, as there is in pulp bleaching for the paper industry.

Sapwood, Heartwood & Decayed Wood

After steam treatment of aspenwood, particularly after shorter treatment times, some chips are clearly less well-cooked than others. This undercooking has been observed, even when there was no possibility that any of the chips had been immersed in condensed water during the steam treatment, and even when the charge of chips was small. It is possible that this apparent difference in response to steam treatment was related to differences between heartwood and sapwood (although these are not well defined in aspenwood), or to the presence of wet pockets. Possible differences in response could have a bearing on the uniformity of treatment and on the best age of wood to be used. Young trees, on which much of the published work has been done, contain little or no heartwood. Also, the slab wood used in

other published studies was all sapwood. The response of old, over-mature, partly-decayed aspenwood, such as that constituting much of the poplar resource in Alberta, has not been adequately studied. In the case of spruce, differences in response may be found for heartwood and sapwood, because of the much greater moisture content of the latter. In addition, because of the well-known refractory nature of sprucewood to pressure impregnation by preservatives, chip size of sprucewood may have an effect on response to steam treatment.

Factors Affecting Accessibility

Enzymatic hydrolysis correlates reasonably well with extent of hemicellulose removal, over a substantial range of hemicellulose contents. However, even after virtually all of the pentosan of aspenwood has been solubilized or destroyed, further steam treatment continues to improve the subsequent rate and extent of enzymatic hydrolysis of the cellulose. Factors other than pentosan removal are therefore involved. Removal of most of the lignin from aspenwood, as in the procedure for holocellulose preparation, also greatly improves enzymatic hydrolysis, even without the removal of pentosan. Crystallinity has been reported to be an impediment to enzymatic hydrolysis. Ball milling reduces crystallinity and increases hydrolysis, but steam treatment increases crystallinity and yet also increases enzymatic hydrolysis. Ball milling and steam treatment, however, both reduce cellulose DP, which may, therefore, be a factor. The extent of swelling decreases irreversibly on drying, as does also the rate and extent of subsequent enzymatic hydrolysis.

All of the above-mentioned factors may well affect enzymatic hydrolysis. It would be desirable, from the point of view of further improving the best substrates available, to know which factors are the most important.

Substrate Characterization

A significant discrepancy is frequently observed between HPLC and anthrone values for glucose. The two methods are in excellent agreement when applied to standard glucose solutions, but, when applied to Klason lignin filtrates in the analysis of steam-pretreated substrates, the HPLC value for glucose is often 5% or more below the anthrone value. This discrepancy occurs even when other sugars, such as xylose and mannose which could interfere with the anthrone method, are virtually absent. It is important to determine the cause of the discrepancy because a low HPLC value obtained in the substrate analysis could explain some of the greater-than-100%-of-theoretical yields obtained on enzymatic hydrolysis.

MATERIALS AND METHODS

Material

Green never-dried aspenwod chips 3.2-4.5 mm in the fiber direction were cut from a freshly felled *Populus tremuloides* tree and were stored frozen in sealed plastic bags. In some experiments, where specified, commercial aspenwood chips were used. These chips were also stored frozen.

Modification of Apparatus for Steam Treatment

The existing two-litre pressure vessel[1] was modified by replacing the Parr-bomb lid with a pneumatically actuated ball valve, for rapid loading. The capability of inserting probes, or other equipment, down through the top of the vessel was retained. In experiments requiring this to be done, the new upper ball valve can be left open, and the Parr-bomb lid can be attached above this ball valve. The probes, or other equipment can then extend down through the open valve. Pressure control switches, with an override capability, have been added to prevent inadvertent opening of the upper and lower ball valves, while the vessel is under pressure.

In order to permit operation, with or without explosion, the cyclone was removed, and the pressure vessel was mounted on a steel platform, to which the bottom flange of the vessel was bolted. For use without the cyclone, a tray could be placed below the steel platform to catch the charge of steam-treated chips which would fall through the lower ball valve of the vessel, and through a circular hole of the same diameter in the platform. Before opening the ball valve, of course, the steam pressure was released slowly through a bleed valve near the top of the vessel. A photograph of the modified pressure vessel, taken before the insulation was applied, is shown in Fig. 1.

An alternative way of treating chips or other material with steam, and of terminating the treatment without an explosion, involved the use of a thin-walled stainless steel canister shown in Fig. 2. When used, the canister was filled with the chips or

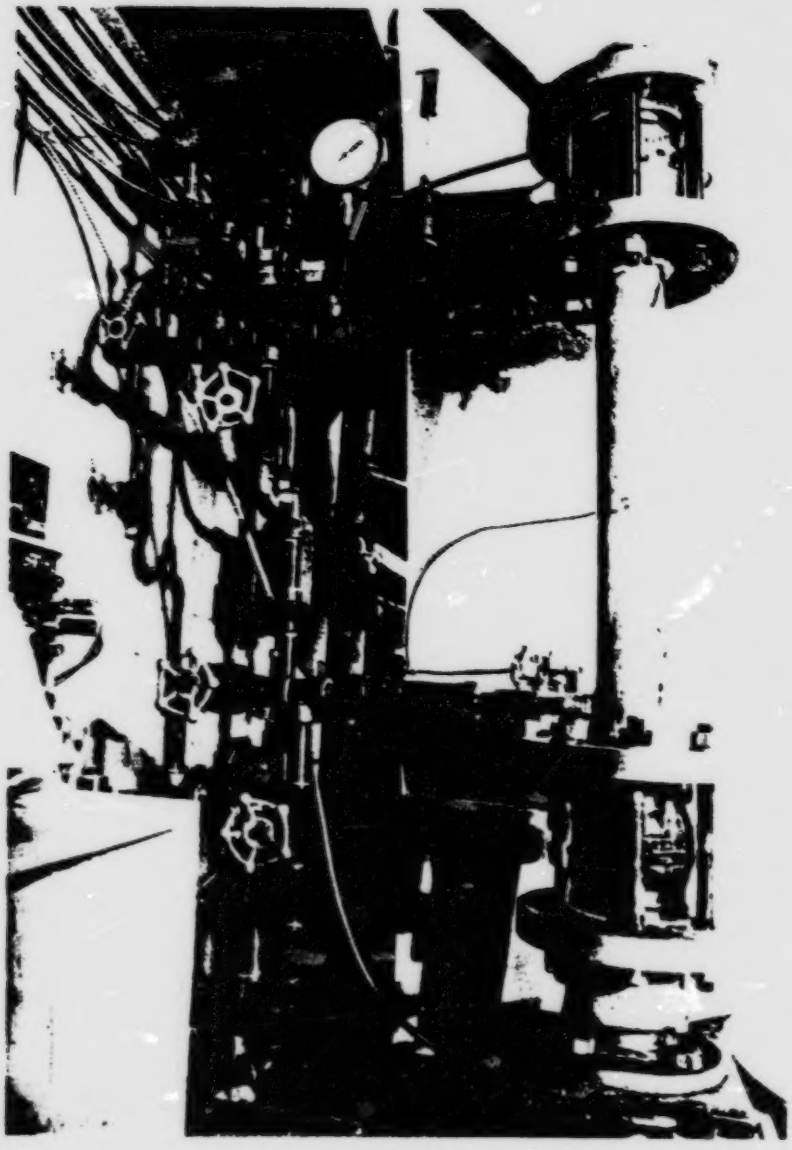


Figure 1. Modified two-litre apparatus for steam treatment

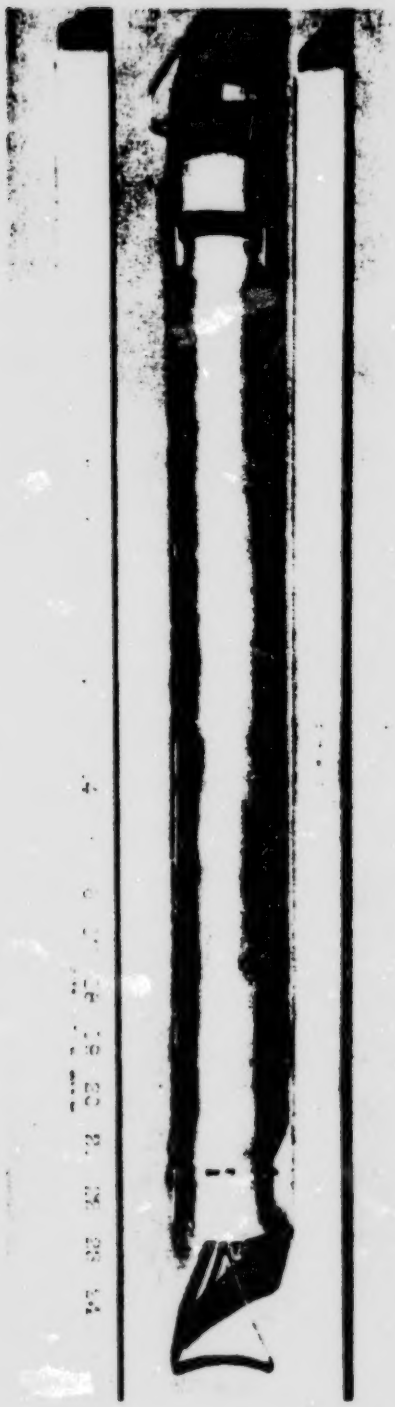


Figure 2. Canister for holding chips during steam treatment without explosion

other material to be treated, and the loosely fitting canister lid (also shown in Fig. 2) was locked in place. An annular space 2.5 mm wide, between the canister and the lid, permitted steam to enter and to leave the canister during pressurizing and during bleed-down. The canister, with its charge of chips was lowered into the preheated pressure vessel through the open upper ball valve. The ball valve was then quickly closed and steam was admitted from the boiler. Spacers, silver-brazed to the outside of the canister, centered it in the pressure vessel, leaving an annular space about 6.4 mm wide between the canister and the vessel wall. The lower end of the canister was supported by a foot, which rested on the closed lower ball valve and kept the lower end of the canister above the level of the lower side arm of the pressure vessel. This sidearm was connected to a steam trap which vented condensate from the vessel and kept the water level from reaching the canister during long treatments.

At the end of the desired time of treatment, the inlet steam line was closed, and a bleed valve just above the lower ball valve was opened, to the extent required to permit the pressure to drop at any desired rate. Finally, the upper bleed valve was opened as well, to ensure that atmospheric pressure had been reached. The upper ball valve was then opened, and the canister, with its contents at 100^{o}C, was removed and was cooled in a pail of cold water. The lower ball valve remained closed.

The canister, of course, was never used when the charge was to be exploded. When the steam treatment was to be terminated with an

explosion, the chips were loaded directly into the two-litre pressure vessel. The cyclone in this case was easily re-attached by moving it close to the pressure vessel, so that the flange, of the cyclone elbow slid under the lower surface of the steel table supporting the pressure vessel. The bolts were then passed through the flange of the pressure vessel, the steel table top, and the flange of the cyclone elbow, clamping the vessel, table and cyclone, together.

Electron Microscopy

Samples of steam-treated aspenwood, which had not been exploded and were still in chip form, were air dried and coated with a conductive film, in a sputter coater, and were observed in a JEOL JSM-35 scanning electron microscope.

Analytical Methods

Pentosan. Pentosan was determined by TAPPI standard method T 223 os-71. In some cases, where other C_5 sugars were essentially absent, pentosan was calculated from the xylose concentration in the Klason lignin hydrolysate, as determined by HPLC.

Lignin. Klason lignin (acid-insoluble lignin) was determined by TAPPI standard method T 222 os-74. Acid-soluble lignin was determined by TAPPI Useful Method 250.

Glucose. During the first year of the contract, glucose in enzymatic hydrolysates was determined by the Glucostat method (23), and during the second year by the Glucostat, the YSI (Yellow Springs Instrument Co.), and HPLC methods. During the third year, the Glucostat and YSI methods were dropped.

Glucose and cellobiose in Klason filtrates were determined, during the second and third years, by HPLC methods. During the second year, the anthrone method was further improved and was subsequently also used on Klason filtrates to determine hexosan hydrolysis products, as glucose. This improved anthrone method is described in detail in Appendix 1. It gives the sum of the glucose equivalents of glucose, cellobiose, cellotriose and higher oligomers. It also includes the glucose equivalent of any mannose or galactose, and oligomers of them.

HPLC Methods. A Bio-Rad HPX 87P column, with a guard column (Bio-Rad, Carbo P) and water as an eluant, was used in some cases when mineral acid was not present, as was the case with the water-solubles fractions of uncatalyzed steam-treated wood. It was also used in some experiments on Klason filtrates, in which case the sulfuric acid was precipitated with barium hydroxide, and an internal standard (erythritol) was used.

When Klason filtrates were analysed, a Bio-Rad HPX 87H column with a guard column (H-cation, Bio-Rad) was usually used, with 0.01N sulfuric acid as eluant. This 87H column was also used for the water-soluble fractions of steam-heated wood, if acetic acid was also to be determined. In a few cases, a BioRad HPX 42A column was used for separation of oligomers. For most of the HPLC analyses, a Varian HPLC system (Model 5000; Varian N.J. USA) interphased with a Varian CDS 401 data system was employed. A refractive index detector (Varian RI-3) was used. The HPLC sample injector (Model 7126; Rheodyne, CA, USA) was fitted with a

20 uL sample loop.

Total Reducing Sugars. Total reducing sugars were determined, during the first two years, by the dinitrosalicylic acid (DNS) method of Miller (24).

Crystallinity Index. The crystallinity index was measured by the method of x-ray diffraction, using a Norelco diffractometer which was run at 40 kV and at 20 mA. Samples were ground and pressed at 11 metric tons to form pellets. The crystallinity index was calculated according to Segal et al (25) using the intensities from the crystalline and amorphous fractions of the cellulose.

Ethanol. Ethanol was determined essentially by the GC method of Saddler et al (26).

Enzyme Preparation

Trichoderma harzianum E58 from the Forintek culture collection was grown on water-washed steam exploded aspenwood (80s, 240°C). The culture filtrate was concentrated by ultrafiltration using a Millipore membrane with a molecular-weight exclusion limit of 10,000. The enzyme preparation was assayed (27) and was diluted to obtain the desired activity. In some cases, where specified, a mixture of commmercial enzymes (Novozyme and Celluclast) was used.

Enzymatic Hydrolysis

The hydrolysis procedure followed in obtaining the data of Table 2 and Fig. 7 was as follows: Test vials, containing the water-washed steam-treated wood at a cellulose concentration of 5% in a

0.5 M sodium acetate buffer at pH 4.6, and a filter paper activity of 15 IU/g of cellulose, were incubated for 2 days at 45°C with swirling at 150 rpm. The required weight of water-washed steam-treated wood, to be added to each test vial, was calculated from the hexosan analysis, in order that the concentration of actual hexosan was 50 mg per mL. The hexosan in the water-washed steamed wood was virtually all cellulose.

The hydrolyses with the much larger enzyme loading, used in obtaining the higher-conversion levels shown in Tables 3, 5 and 7 and in Fig. 9, 10 and 11, were done at total substrate concentrations of 2% and 5%. In all subsequent experiments, the enzyme loading, as well as concentration of substrate, were expressed on total substrate, and not on cellulose content of the substrate. The initial specified concentrations, of 20 or 50 mg/mL, therefore included the weights of pentosan and lignin as well as of cellulose. Calculated conversions, as percentages of the theoretical glucose yields, were based on the hexosan analyses of the substrates.

Simultaneous Saccharification and Fermentation

Test vials containing water-washed steam-treated aspenwood at a cellulose concentration of 5%, a yeast extract concentration of 0.5% and a yeast inoculum of 5%, in 0.05 M sodium acetate buffer at pH 4.6, were incubated for 2 days at 37°C with swirling at 150 rpm.

RESULTS AND DISCUSSION

Pyrolysis with Dry Steam (Objective 1)

A few additional pretreatments of aspenwood wafers with dry (superheated) steam were done by passing the dry 276°C steam at atmospheric pressure through a pipe loosely packed with the air-dry wafers. The results are not recorded here in detail because they simply confirmed our previous conclusions (1). Under these dry conditions, the pentosan fraction was gradually destroyed with very little solubilization in either water or in dilute alkali, and with very little increase in the yield of glucose, or other reducing sugars, on subsequent enzymatic hydrolysis. Heat transfer was less uniform with this high-temperature dry steam, than with high pressure saturated steam. The wafers rose quickly in temperature to 100°C and became dry, after which no condensation occurred. The rapid entry of steam into the wood, and release of the latent heat of vaporization inside the wood, did not occur. As a result, scorching of the surface of some of the wafers resulted, and appeared to be related to the uneven flow of the dry steam between the wafers. Surfaces exposed to wider channels through the bed apparently were heated more rapidly.

Attempts were made to achieve more uniform heating. Smaller charges of thinner wafers were used, and the wafers were positioned to obtained more even heating. Also, the steam flow was more carefully controlled, and the temperature at different locations was more extensively monitored. The results, however, were essentially as before. Little solubilization of pentosan in

either water or dilute alkali was obtained. In the light of Grethlein's pore-size studies, it appears that the failure to remove pentosan, during dry pyrolysis, meant that adequate pore formation did not occur.

Determination of Conditions Minimizing Pyrolysis (Objective 2)

Pentosan Solubilization and Destruction

Because of the conflicting reports in the literature, on the effect of steam temperature on the destruction of the pentosan fraction of wood, a series of treatments was made at the relatively low temperature of 190°C, for comparison with the results obtained at 240°C.

The results are shown in Table 1 and in Fig. 3. All data are expressed as percentages of the dry equivalent weight of the wood originally put into the two-litre steaming apparatus, in order to facilitate comparison. Compositions of the steam-treated wood, before and after water washing, can easily be reconverted back to a steamed-wood or washed-steamed-wood basis by dividing by the appropriate percent-recovery values also listed in Table 1. Times of heating in steam at 190°C have been chosen, in order that roughly the same range of pentosan destruction is covered, as is covered by the data for 240°C. The time scale in Fig. 3, for the 190°C data, has been adjusted by the factor of 2^5 (i.e. by 32), to take into account the approximate doubling of the rate of pentosan solubilization for each of the five 10-degree increases in temperature between 190 and 240°C.

From Fig. 3 it can be seen that the data points, for 190 and

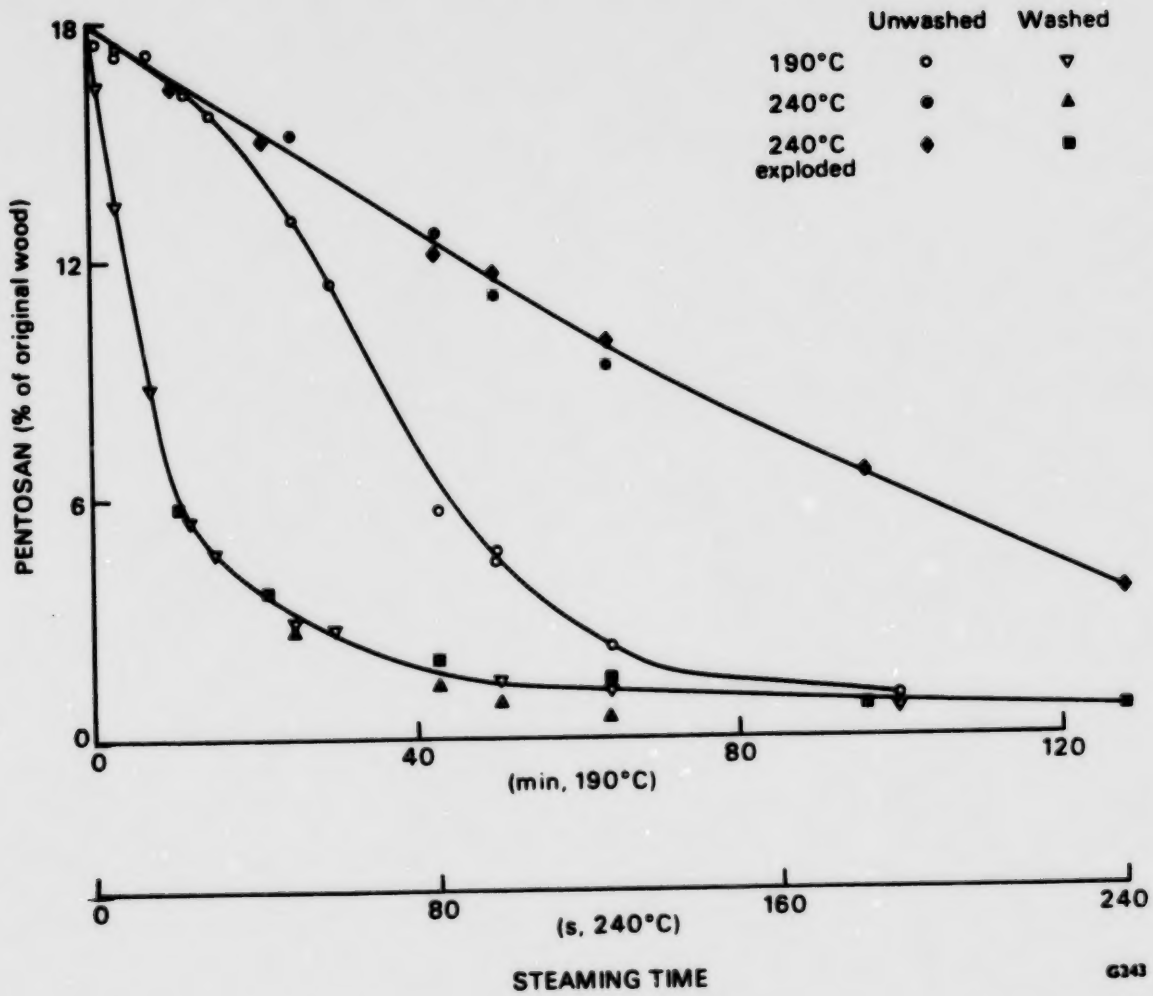


Figure 3. Effect of steam temperature (190 or 240°C) on the pentosan content of steam-treated aspenwood, before and after water washing

Table 1. Comparison of Recovery and Composition of Steam-heated Wood[1], before and after Water-Washing, for Treatments at 190°C without Explosion, and at 240°C with Explosion

Time of treatment (min)	Steamed Wood before washing		Steamed Wood after Washing			
	Recovery (% of original wood)	Pentosan content (% of original wood)	Recovery (% of original wood)	Composition (% of original wood) Pentosan	Hexosan	Lignin[2]
190°C, no explosion						
12	96.2	16.3	77.4	5.6	47.5	21.5
25	92.7	13.0	73.6	2.7	46.5	21.9
50	83.9	4.6	73.3	1.3	45.6	24.7
100	83.3	0.9	77.0	0.7	44.5	27.8
240°C, with explosion						
0.33	95.5	16.3	76.9	5.8	47.4	19.9
0.67	94.8	15.0	75.7	3.6	49.0	21.8
1.33	93.3	12.2	75.2	1.9	48.7	23.2
2.00	89.9	9.9	71.8	[illegible]	48.9	23.3
3.00	84.2	6.6	69.6	0.7	45.0	24.2
4.00	78.5	3.5	65.9	0.7	42.9	23.5

1 Composition of original aspenwood: pentosan, 18.4; hexosan, 49.3; lignin, 24.4 (analysis on extractives-containing wood)

2 Lignin is "apparent lignin" and includes "pseudolignin" which is produced during degradation of pentosan

240°C, lie reasonably well along the same lowermost curve. This curve, for insoluble pentosan remaining after steam treatment and water washing, indicates at least as good a reduction in insoluble pentosan content after treatment at 190°C without explosion, as after treatment at 240°C with explosion. Also included on this curve, are data point for 240°C without explosion.

The uppermost curve of Fig. 3 shows the total amount of pentosan (soluble plus insoluble), which survived steam treatment and explosion at 240°C. Also plotted along this curve are corresponding data at this same temperature, but without explosion. The similarity of these two sets of data shows the equivalence of the two treatment conditions and the lack of any apparent effect of the explosion on pentosan destruction. This conclusion contradicts earlier patent claims (9,10).

The middle curve of Fig. 3 shows the total amount of pentosan (soluble plus insoluble), after treatment at 190° without explosion. This curve falls much more steeply than does the uppermost curve at 240°C, showing that, for the same amount of pentosan removal, more destruction of pentosan occurred at the lower temperature of 190°C. It is apparent, therefore, that the activation energy for the desirable process of pentosan hydrolysis, is greater than that for the undesirable process of pentosan destruction. In the absence of added acidic catalysts, the greatest survival of pentosan should occur for steam treatment at the highest possible temperature, consistent with a

uniform heating of the wood.

The amount of water-soluble pentosan surviving the steam treatment is given by the vertical distance between the curves of Fig. 3. For treatment at 240°C, this distance is the distance between the uppermost and lowermost curves, whereas at 190°C it is between the middle and the lowermost curves. It can be seen that, at the shortest treatment times at the two temperatures, the yields of water-soluble pentosan were not much different. However a treatment comparable to 80s at 240°C would probably be required. In earlier work, it was found that the yield of glucose, obtained on enzymatic hydrolysis of aspenwood, increased steeply with time of steam treatmen at 240°C until after about 80s, when it tended to "level-out". At the corresponding time of 42.6 min at 190°C, it can be seen that the yield of water-soluble pentosan was slightly less than half of that at 80s at 240°C. Much greater losses of pentosan would, therefore, probably result from steam treatment at 190°C, rather than at 240°C, unless some other measures were taken (such as the addition of an acidic catalyst).

Recovery of Steam-Treated Wood

Support for the pentosan data of Fig. 3 can be seen in the corresponding recovery data of Fig. 4, for steam-treated wood both before and after water washing. The greater loss of total (soluble plus insoluble) pentosan, in the case of wood steam-treated at 190° is reflected in the corresponding steeper drop in total recovery of steam-treated wood, much of the destroyed pentosan being lost as volatiles. The "levelling-out" of this

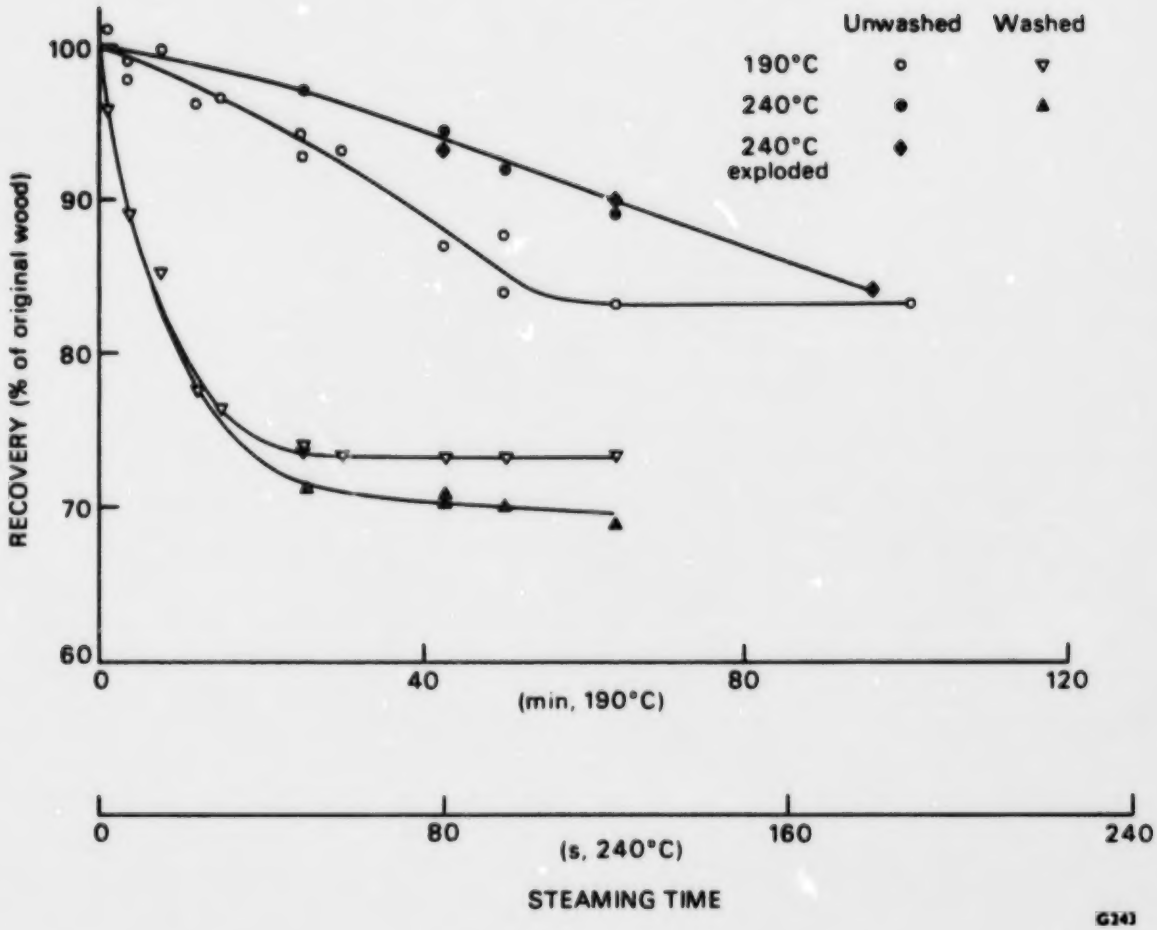


Figure 4. Effect of steam temperature (190 or 240°C) on the weight of steam-treated aspenwood recovered from the steam treatment, before and after water washing

curve also occurs at the same time as does that for the total pentosan, which corresponds to the time required for total destruction of all the pentosan. After water-washing, however, recovery of wood steam-treated at 190°C is seen to have been a few percent greater than that at 240°C, reflecting the greater content of pseudolignin produced by the greater destruction of pentosan at 190°C. Much of the material, which at 190°C was weighed as pseudolignin, was, at 240°C, washed out as water-soluble pentosan.

The recovery data which were obtained after water-washing, and which are presented in the lower pair of curves of Fig. 4, were all obtained by the use of the canister, without explosion. Possible losses in the cyclone were therefore avoided, and an accurate comparison was assured. In the uppermost curve of Fig. 4 for total recovery after steam-treatment at 240°C, data are included both for canister-treated wood, and for wood treated without the canister and exploded into the cyclone. In the latter case, care was taken to cool the cyclone, to prevent drying of any of the exploded product during recovery. No significant difference can be seen between the exploded and not-exploded results.

Pseudolignin Formation

The formation of greater amounts of pseudolignin during steam treatment at 190° than at 240°C, is shown in Fig. 5. The apparent lignin values plotted in Fig. 5 consist of apparent Klason lignin plus acid-soluble lignin. The apparent Klason

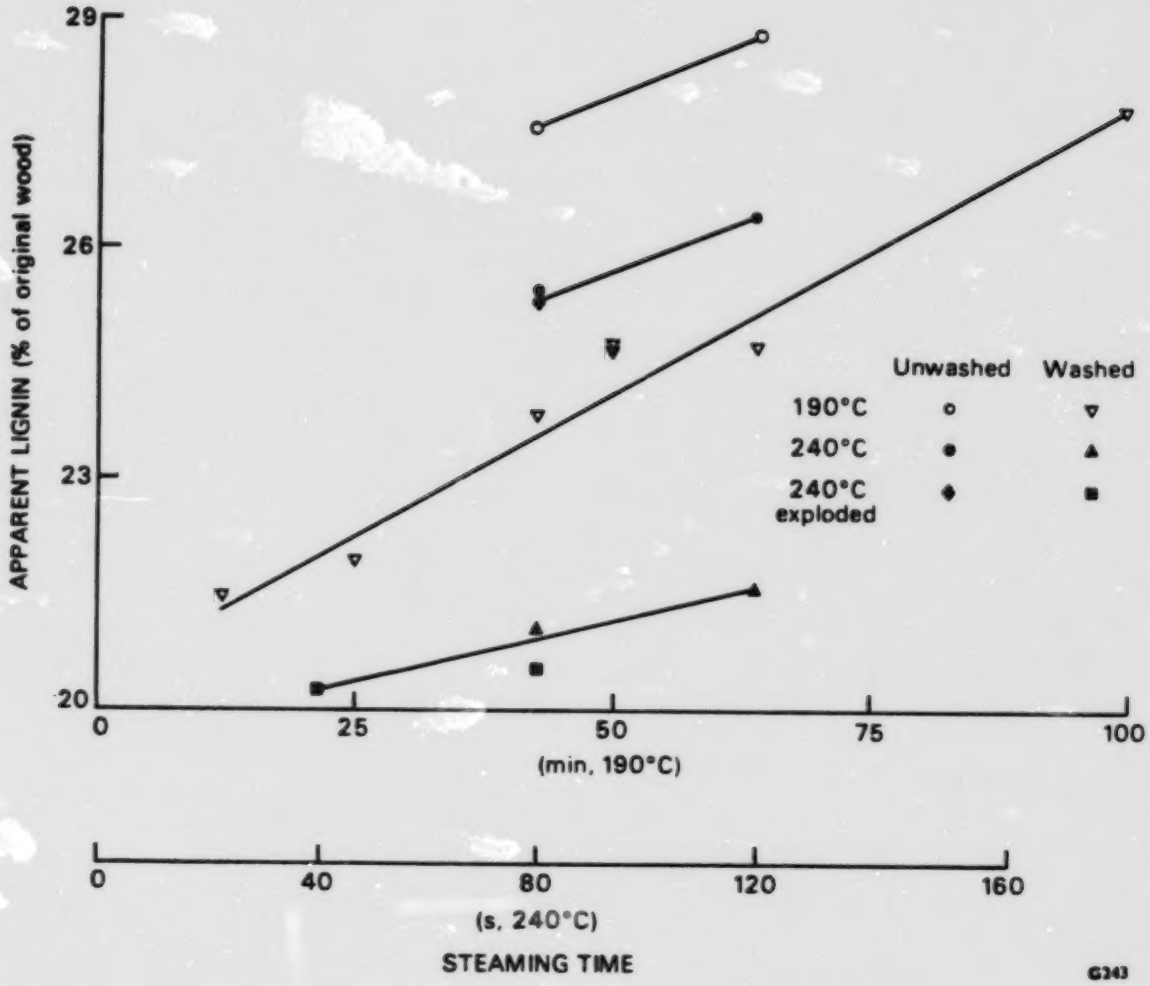


Figure 5. Effect of steam temperature (190 or 240°C) on the apparent lignin content of steam-treated aspenwood, before and after water washing

lignin consists of true Klason lignin, plus pseudolignin formed largely from degraded pentosan and weighed together with the Klason lignin. For comparison, all apparent lignin values, plotted in Fig. 5, are expressed as percent of original wood. Greater contents of apparent lignin can be seen after treatment at 190° than after treatment at 240°C, both before water washing (upper pair of curves), and after water washing (lower pair of curves). Approximately 12% of the lignin of the original aspenwood is rapidly solubilized (in water) during the early stages of steam treatment, after which any increase in apparent lignin is the result of pseudolignin formation.

Hexosan Survival

The hexosan contents, remaining after water-washing of aspenwood steam-treated at 190°C, are shown in Fig. 6 on an original wood basis. The declining hexosan content, with increased time of steam treatment, results mainly from a solubilization of cellulose and other hexosan, rather than from destruction of these materials. The more rapid decline early in the treatment (which also occurs at 240°C), results in large part from a rapid dissolution of much of the glucomannan fraction. This fraction, which is about half mannose and half glucose, represents about 3% of the original aspenwood. The total hexosan content of the original wood accordingly dropped from 49.3% to 47.5% within the first 12 minutes, but then dropped only another 1.9% to 45.6% after a total treatment of 50 minutes. It is unlikely that a treatment as long as 50 minutes at 190°C would be used in practice. Loss of cellulose at 190°C, therefore, should not

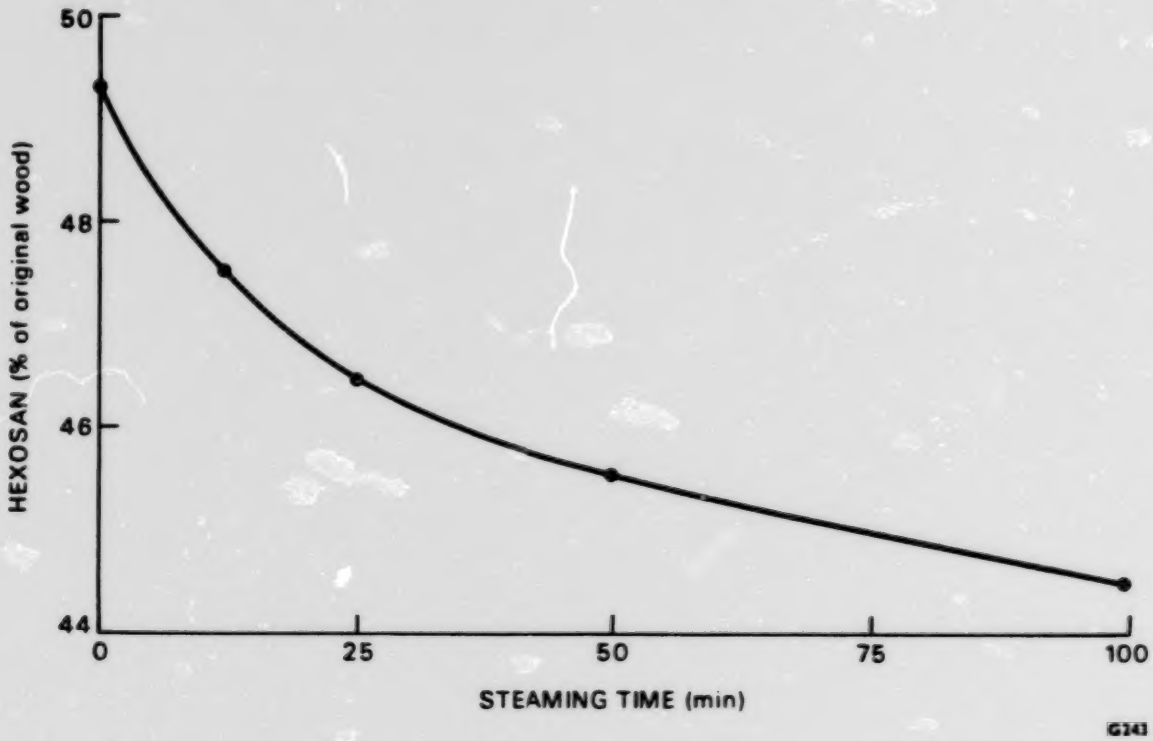


Figure 6. Effect of steaming time at 190°C on the hexosan content of aspenwood, after water washing

constitute a problem.

Enzymatic Hydrolysis

In order to compare the enzymatic hydrolyzability of aspenwood steam-treated at 190°C without explosion, with the hydrolyzability of wood steam-treated at 240°C and exploded, the data presented in Table 2 and in Fig. 7 and 8, were obtained. The time scales have been adjusted as described for Fig. 3, and a curve showing the residual pentosan content of the water-washed materials has been included, to permit comparisons at equal levels of pentosan removal. The similarity of the two glucose-yield curves of Fig. 7 for enzymatic hydrolysis, and of the two ethanol-yield curves of Fig. 8 for combined hydrolysis and fermentation, indicates that the steam treatment at 190°C without explosion was at least as good as was the treatment at 240°C with explosion, in conferring enzymatic accessibility to the cellulose.

In order to make an additional comparison of the enzymatic hydrolysis at a higher conversion level, a much larger loading of enzyme preparation (also from T. harzianum E58) was used. Also, the hydrolysis was done at a lower substrate concentration. The results are presented in Table 3 and in Fig. 9.

In this experiment, only two samples were compared. They were: 1) aspenwood steam-treated at 240°C for 80 seconds, with explosion, and 2) aspenwood steam-treated at 190°C for 25 minutes without explosion. Both were water-washed, after which they had pentosan contents of 2.2 and 3.6% respectively, and total

Table 2. Comparison of Enzymatic Hydrolysis[1] and of Combined Hydrolysis and Fermentation (CHF) of Water-Washed Steam-Heated Wood, for Treatments at 190°C without Explosion, and at 240°C with Explosion

Time of treatment (min)	Enzymatic Hydrolysis[2] Reducing sugars (mg/mL)	Glucose (mg/mL)	CHF Ethanol (mg/mL)
190°C, no explosion			
12	11.8	8.4	5.0
25	13.4	10.7	6.4
50	12.9	10.9	6.9
100	14.3	12.2	7.4
240°C, with explosion			
0.33	11.1	6.3	3.9
0.67	12.5	8.9	5.1
2.00	11.5	9.1	5.8
3.00	12.7	9.6	6.4
4.00	11.9	9.0	6.0
Solka floc, no treatment			
0	32.6	25.0	13.0

1 Enzyme preparation of relatively low activity

2 Substrate concentration selected to be equivalent to 50 mg/mL of hexosan (hexosan was almost entirely cellulose)

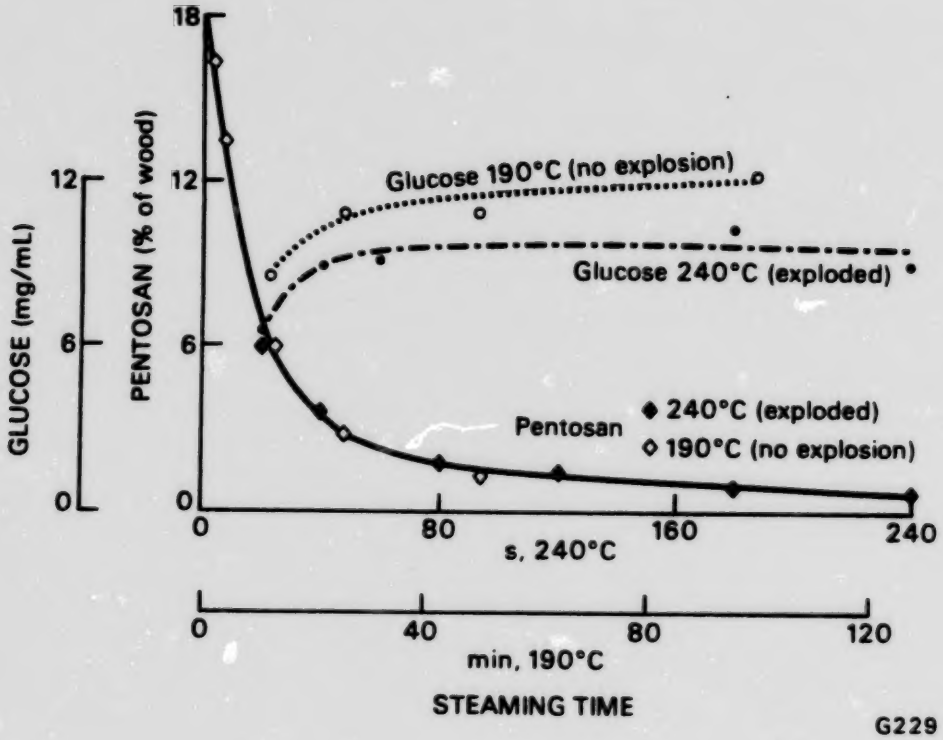


Figure 7. Comparison of enzymatic hydrolysis with residual pentosan content, for water-washed steam-treated aspenwood (190°C, no explosion, and 240°C, with explosion)

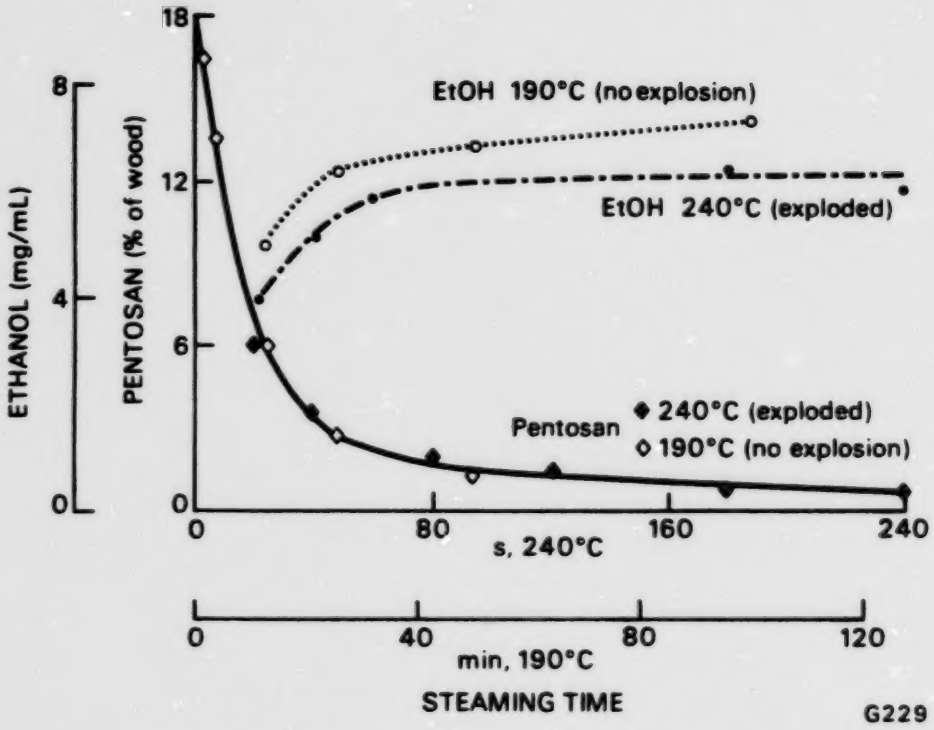


Figure 8. Comparison of combined hydrolysis and fermentation with residual pentosan content, for water-washed steam-treated aspenwood, (190°C, no explosion, and 240°C, with explosion)

Table 3. Comparison of Enzymatic Hydrolysis to Higher Conversion Levels[1], with a Much Larger Enzyme Loading, of Water-Washed Steam-Heated Wood, for Temperatures at 190°C without Explosion, and at 240°C with Explosion

Treatment	Initial Conc. Total substrate (mg/mL)	Initial Conc. hexosan (mg/mL)	Hydrolysis time (hours)	Reducing sugars (mg/mL)	Glucose conc. (mg/mL)	Glucose % of theoretical[2]
190°C, 25 min, no explosion	20	13.2	6	6.4	6.3	43
			24	9.0	9.6	66
			48	10.0	10.8	74
	50	32.9	6	11.4	13.0	36
			24	21.8	23.6	65
			48	23.3	23.6	65
240°C 80s, with explosion	20	13.7	6	8.0	7.9	52
			24	10.4	10.4	69
			48	10.6	11.3	74
	50	34.2	6	13.8	14.6	38
			24	24.5	24.8	65
			48	28.0	26.8	70

1 Higher conversion than in Table 2; higher concentration of enzyme preparation; more dilute substrate (20 mg/mL)

2 Glucose yields plotted in Fig. 9

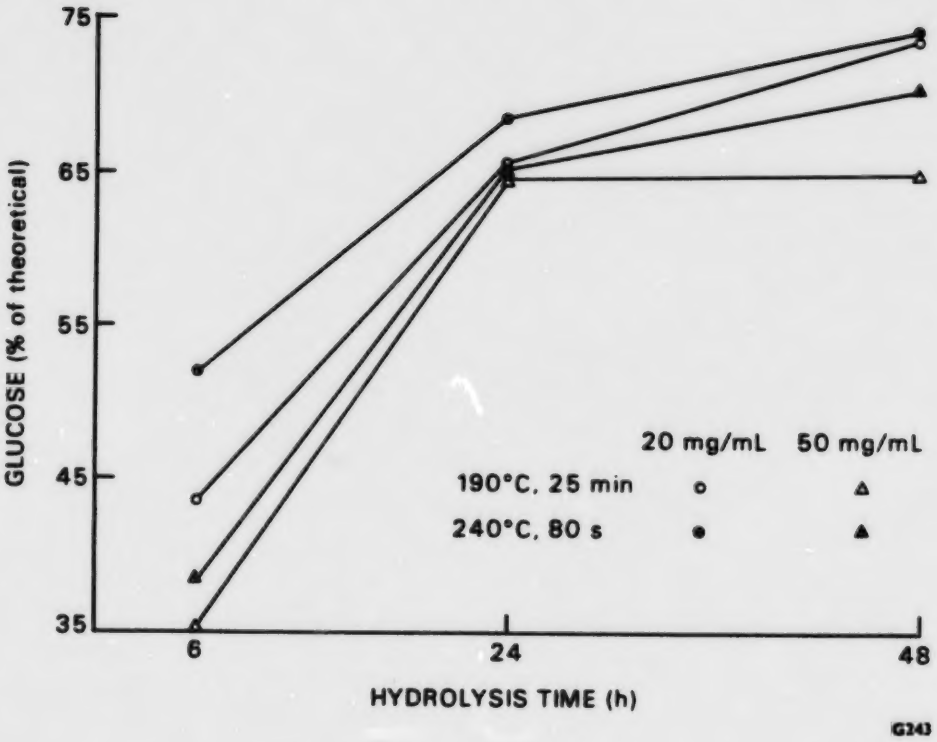


Figure 9. Comparison of the enzymatic hydrolysis of aspenwood, heated for 25 min in steam at 190°C without explosion, with that of aspenwood heated for 80s in steam at 240°C with explosion

apparent lignin contents of 29.4 and 30.6% respectively. As can be seen in Fig. 7, after 80 seconds in steam at 240°C, both the pentosan-removal and the glucose-yield curves had begun to "level-off". From Fig. 3, however, it can be seen that after 25 minutes at 190°C, the pentosan content, after water-washing, was still falling more steeply. This 25-minute sample was selected, although it was apparently less-completely cooked, because a longer time would have resulted in excessive pentosan destruction.

Fig. 9 indicates that, at the lower total substrate concentration of 20 mg/mL, there was little or no difference in glucose yield between the 240 and 190°C samples after both 24 and 48 hours of hydrolysis, although initially the 240°C sample apparently hydrolyzed more rapidly. At the higher total substrate concentration of 50 mg/mL, there was no significant difference after both 6 and 24 hours, although after 48 hours the 240°C sample apparently increased relatively more in glucose yield. Any difference appears to be in favour of the more completely cooked 240°C sample as might have been expected.

An additional comparison of the enzymatic hydrolysis of the two water-washed steam-treated products (190°C, 25 min, no explosion, and 240°C, 80s, with explosion) was completed during the second year of the contract. This comparison which was made with a mixture of the commercial enzymes, Novocellulast and Novozyme, also showed little difference between the two substrates. These data are included in Table 5, in the following section on

secondary treatments with hydrogen peroxide.

Secondary Treatments

Hydrogen Peroxide

If the relatively low steam temperature of 190°C were to be used commercially, and if excessive pentosan loss were to be avoided by the use of relatively short steaming times (which were only marginally adequate for the development of cellulose accessibility), then some additional secondary treatment might be considered. Oxidation with hydrogen peroxide was investigated as such a treatment. The water-washed steam-treated sample, described above in the immediately preceding two paragraphs, which had been treated at 190°C for 25 minutes, was extracted with dilute alkali to remove much of the lignin, and was then subjected to treatment with hydrogen peroxide. Two levels of treatment were applied. The first was essentially as described by Gould for treatment of corn stalk residues for fodder. The second was with only one-tenth as much hydrogen peroxide. Both were at room temperature and at atmospheric pressure. Details of the treatments are shown in Table 4, together with the relevant changes in chemical composition. The results of enzymatic hydrolysis are presented in Table 5 and in Fig. 10.

Fig. 10 shows no significant change in the extent of hydrolysis as a result of the alkali extraction. The peroxide treatment, however, produced a substantial increase. Most of the beneficial effect was already obtained at the lower level of peroxide addition, where the yield of glucose apparently rose from 77% to virtually 100% of theoretical in the case of the lower substrate

Table 4. Effect of Steam-Heating, Water-Washing, Alkali-Washing and Peroxide Treatments on Chemical Composition of Aspenwood

	Equivalent dry weight (g)	Composition: Analyses (%) P[1]	H[2]	L[3]	Original wood basis P[1]	H[2]	L[3]
Aspenwood	100.0	18.4	49.3	24.4	18.4	49.3	24.4
steam treatment 190°C 25 min ↓							
steam-heated aspenwood	94.3						
water wash ↓							
water-washed steamed wood	74.0	3.6	63.1	30.6	2.6	46.5	22.6
alkali wash ↓							
alkali insoluble steamed wood	59.0	3.8		16.1	2.2		9.5
full peroxide treatment ↓							
Pulp #1	54.7	3.8		10.0	2.1		5.5
1/10 peroxide treatment ↓							
Pulp #2	57.3	3.9		13.5	2.2		7.7

[1] P - pentosan

[2] H - hexosan

[3] L - total apparent lignin (i.e. true Klason lignin, acid-soluble lignin and pseudolignin)

Table 5. Effect of alkali washing and peroxide treatment of water-washed steam-treated aspenwood[1], on enzymatic hydrolysis

Substrate	Conc. of total substrate[2] (mg/mL)	Glucose yield (% of theoretical[3]) E58 6h	E58 24h	E58 48h	NOVO[4] 24h	NOVO[4] 48h
190°C, 25 min						
water-washed	20	43.8	66.2	74.3	68.4	89.1
	50	36.0	65.2	65.2	39.5	60.0
alkali-washed	20	46.6	66.9	76.6	68.9	88.1
	50	37.7	66.0	66.2	36.8	55.3
peroxide pulp #1	20	61.3	77.2	98.9	74.0	102.1
	50	45.0	79.7	85.0	45.6	72.1
peroxide pulp #2	20	51.0	77.8	99.6	73.3	96.3
	50	38.3	74.6	81.6	40.0	57.1
240°C, 80s (with explosion)						
water-washed	20	52.9	69.6	75.4	71.5	85.6
	50	39.0	66.3	71.6	38.2	61.8

1) sample preparation and analysis described in Table 4
2) total substrate (cellulose, hemicellulose and lignin)
3) theoretical yield based on cellulose and other glucan in original input wood
4) mixture of Novocelluclast and Novozyme, 19 FPU per gram substrate in 50 mg/mL case; 47.5 FPU/g in 20 mg/mL case

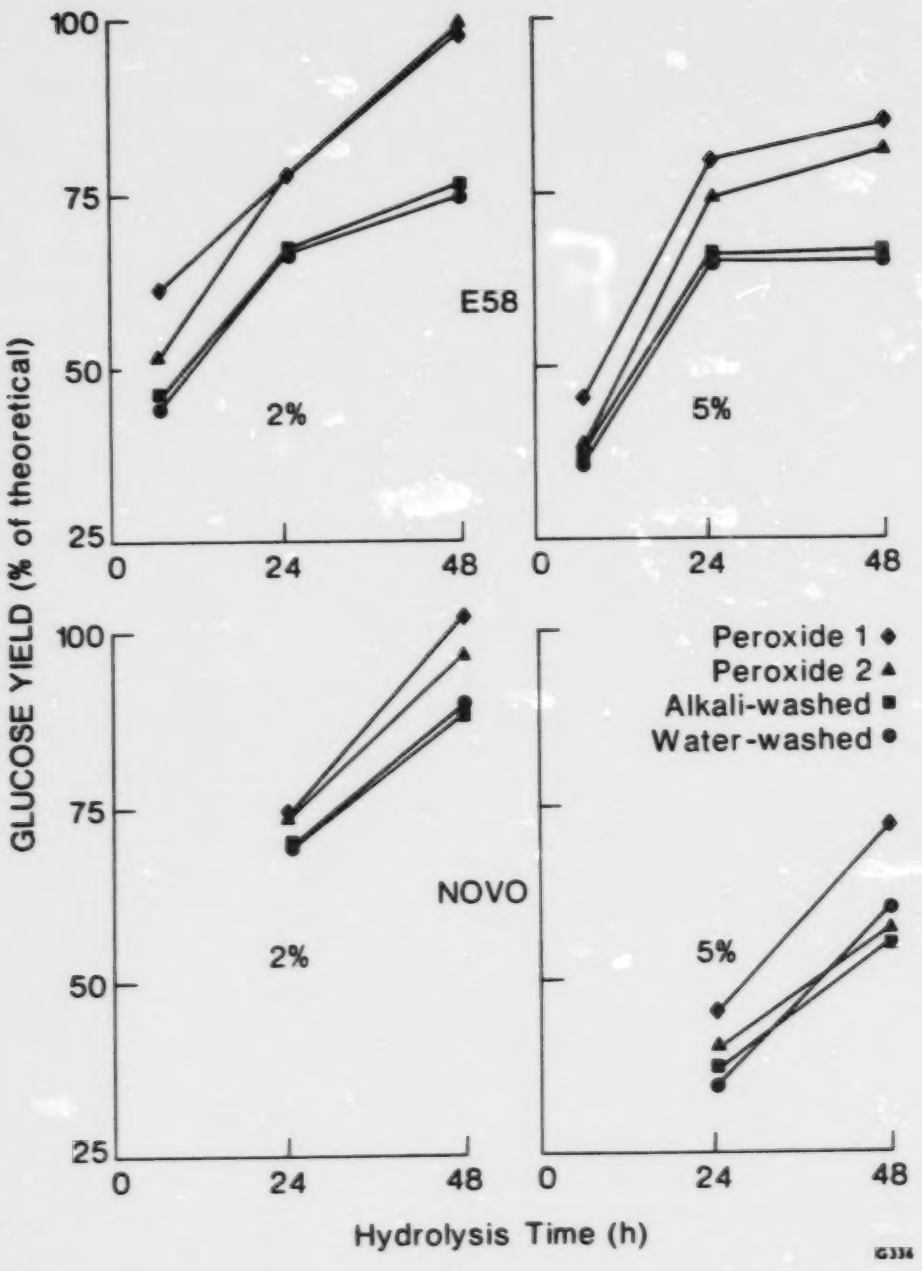


Figure 10. Effect of alkali extraction and treatment with hydrogen peroxide on enzymatic hydrolysis of water-washed steam-heated aspenwood (190°C, 25 min, no explosion) at 2% and 5% substrate concentrations, with E58 and Novo enzymes (substrate overloaded with enzyme in E58 case)

concentration. Moreover, this improvement was obtained even though the peroxide treatment (as shown in Table 4) had left the pentosan content unchanged, and had decreased the lignin content by only 1.8% (original wood basis). This improvement in hydrolysis yield is remarkably similar to that observed earlier by Forintek for a secondary treatment with acidified chlorite, where the lignin content was also only slightly reduced by the almost identical amount of 1.6%. The peroxide treatment, however, has the advantage of being applied at an alkaline pH, such as already prevails after the extraction of lignin, and also of not producing toxic environmentally undesirable chlorolignin break-down products. By applying the peroxide treatment after an alkaline extraction, not only is the bulk of the lignin recoverable as a by-product, but also the consumption of peroxide is apparently reduced. Gould has already shown that, in the treatment of corn-stalk residue (without any preliminary extraction), the unconsumed hydrogen peroxide in the effluent from one treatment can be reused after addition of fresh hydrogen peroxide.

During the second year of this contract, Dr. Schwald of the Institute for Radiochemistry, University of Innsbruck, Austria, was on sabbatical at Forintek. During this time he collaborated with Forintek in a more complete examination of peroxide treatment as a follow-up to steam pretreatment. The most striking results are shown in Fig. 11, 12 and 13. These figures all show that treatment with hydrogen peroxide, after the alkali-soluble lignin had been removed as in the earlier experiments,

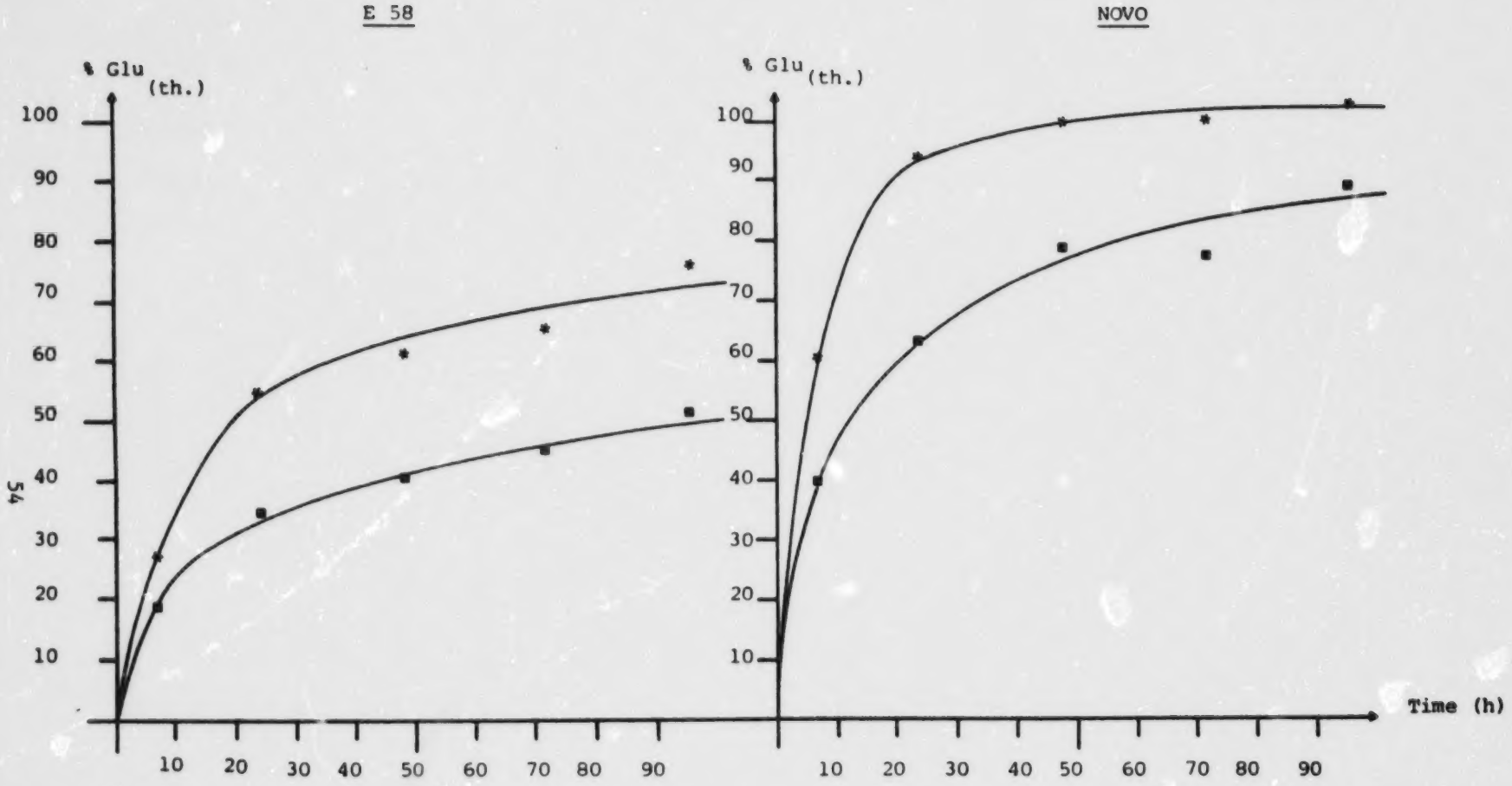


Figure 11. Enzymatic hydrolysis of steam heated (80 s/240°C) aspenwood without posttreatment (SHA-WIA), ■ and with posttreatment(SHA-WIA/H_2O_2), * using 2 different enzyme-preparations; glucose concentrations in % of theoretical plotted against hydrolysis time in h.

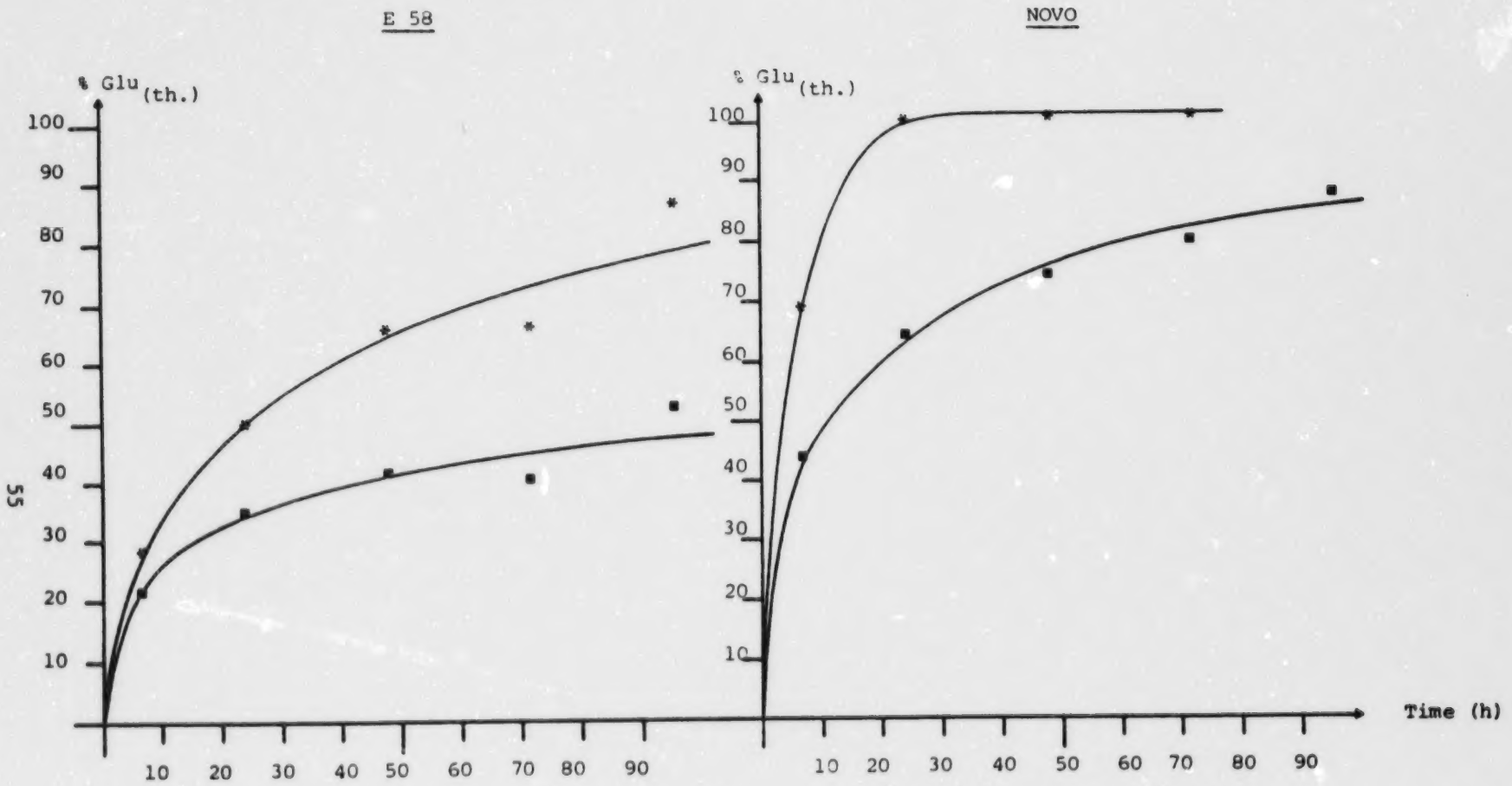


Figure 12. Enzymatic hydrolysis of the steam heated (25 min/190°C)aspenwood without posttreatment (SHA-WIA), ■ and with posttreatment (SHA-WIA/H_2O_2), * using 2 different enzyme preparations; glucose concentrations in % of theoretical plotted against hydrolysis time in h.

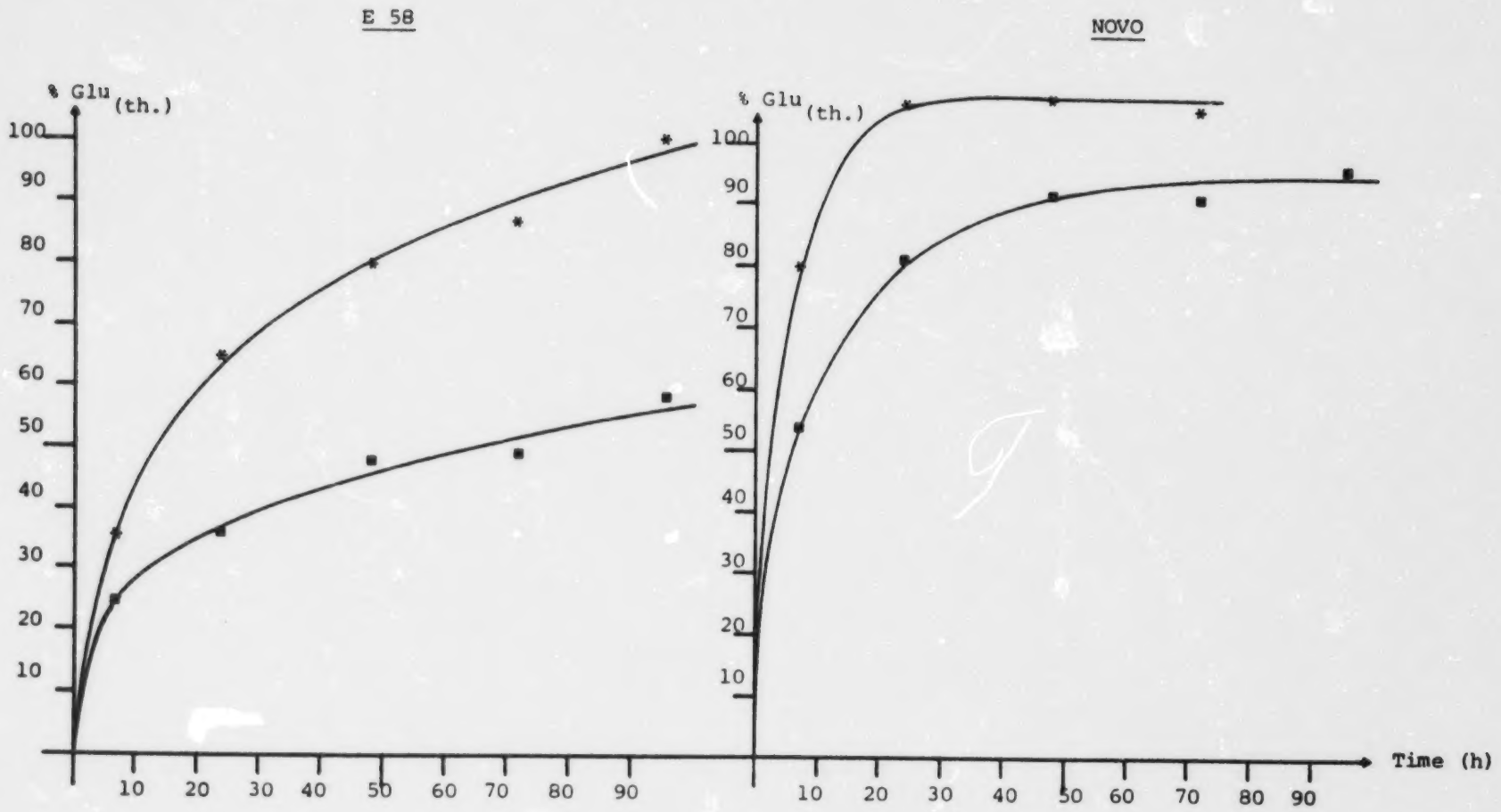


Figure 13. Enzymatic hydrolysis of steam heated (50 min/190°C) aspenwood without posttreatment (SHA-WIA), ■ and with posttreatment (SHA-WIA/H_2O_2), * using 2 different enzyme preparations; glucose concentrations in % of theoretical plotted against hydrolysis time in h.

resulted in substantial improvements in enzymatic hydrolysis. The improvements were shown after steam treatment for 80s at 240°C, and after steam treatments at 190°C for 25 min and for 50 min. All of these treatments were without explosion. Furthermore, the improvements were shown with two different enzyme preparations, in each case.

It is interesting that both of these preparations were used at 25 IU of filter paper activity per gram of oven-dry substrate, as determined using DNS. Also the level of β-glucosidase, as measured by hydrolysis of salicin, was adjusted to be approximately equal in the two enzyme preparations. Nevertheless, hydrolysis with the mixture of commercial enzymes (Celluclast/Novozyme) was faster and more complete than that with the E58. HPLC analysis of the enzymatic hydrolysates also showed higher levels of cellobiose in the E58 hydrolysates. It appears, therefore, that the cellobiase activity of the Celluclast/Novozyme mixtures was higher than that of the unsupplemented E58. Equal abilities to hydrolyze salicin does not, therefore, necessarily mean equal abilities to hydrolyze cellobiose.

In subsequent work, not reported here in detail, it was found that, when HPLC (rather than DNS) was used to determine the amount of glucose produced, the actual number of filter paper units per gram of substrate in the above experiment had been 15.6 in the case of Celluclast-Novozyme, and only 9.4 in the case of E58. These values were in marked contrast to the apparently equal loadings of 25FPU/g as determined with DNS. Similarly,

when the β-glucosidase activity was measured, using cellobiose and HPLC (rather than salicin), the activities were found to have been 35 IU/g in the case of Celluclast-Novozyme, and only 17 IU/g in the case of E58. These values, also, were in marked contrast to the almost equal activities of 24 and 26, respectively, determined with salicin. Furthermore, when equal filter-paper and β-glucosidase loadings of Celluclast-Novozyme and E58 were used (based on these new values), virtually identical hydrolysis curves were obtained, showing that the earlier assays with DNS and salicin had been incorrect.

The rate of enzymatic hydrolysis was much faster after peroxide treatment, especially with the Celluclast/Novozyme mixture where adequate cellobiase was present. In the case of the 190°C treatment for 25 min, the hydrolysis yield, after only 24h increased from about 64% of theoretical (without peroxide) to virtually 100% (with peroxide), where it remained unchanged until after at least 72h.

The effect of peroxide treatment directly on the SHA-WI, without first removing the alkali-soluble lignin, was also examined. Enzymatic hydrolysis, without first removing the alkali-soluble lignin, was at least as good as, or better than that after alkali extraction followed by peroxide treatment. Consumption of peroxide was, of course, much greater in the absence of alkali extraction, and a lignin by-product was not available. Residual lignin content after peroxide treatment (without alkali extraction) was at least as low as that after peroxide treatment

preceded by alkali extraction. The use of a peroxide treatment without first removing much of the easily oxidizable lignin (as in an alkali extraction), would, of course, be too costly to be considered commercially.

During this work, it was observed that, except for very short steaming times, enzymatic hydrolysis of SHA-WIA (i.e. after alkali washing) was initially slower than that of SHA-WI (i.e. after washing with water only, and not with alkali). These comparisons were made at equal enzyme loadings, the loadings being expressed as filter paper units per gram of total substrate (i.e. per gram of SHA-WI or per gram of SHA-WIA), and not per gram of cellulose present in the substrate. Examples of this effect are shown in Fig. 14, for enzyme loadings of 35 IU of filter paper activity. The enzyme in this case was E58. The substrates (SHA-WI and SHA-WIA) had been obtained, after steam treatment, in the yields listed in Table 6. The corresponding chemical analyses are given in Table 7.

Although these data might appear to indicate that alkali washing altered the substrate in some way which reduced its accessibility to the enzyme system, (and, in fact, some discussion as to how this adverse effect could be avoided has appeared among the I.E.A. COSY exchanges), this conclusion is probably incorrect. Table 7 shows that the cellulose (or other glucan) contents of the SHA-WIA substrates were significantly higher than those of the corresponding SHA-WI substrates. The enzyme loadings for the latter were therefore significantly lower, if expressed per gram of cellulose.

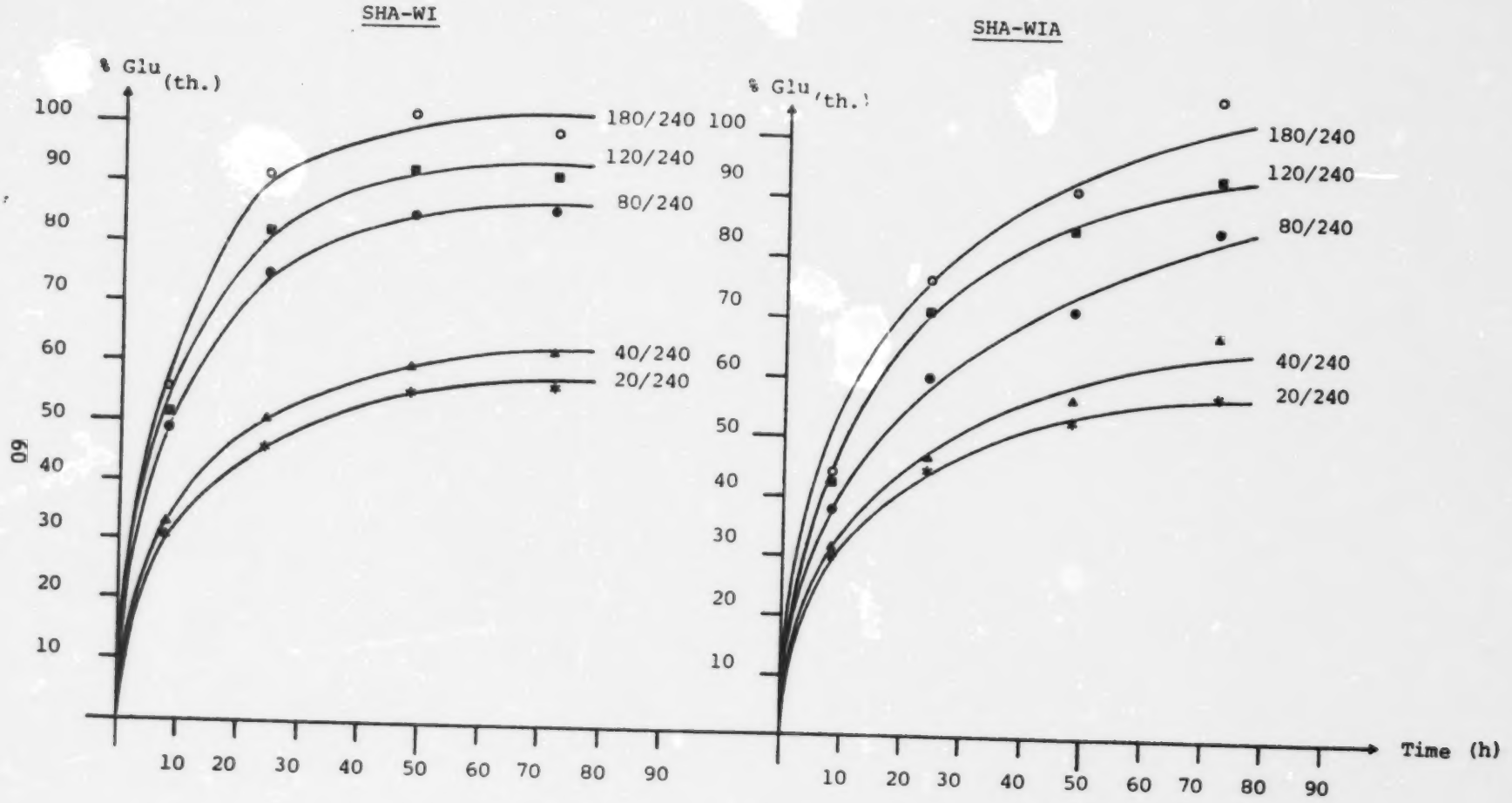


Figure 14. Course of the enzymatic hydrolysis of steam treated samples from aspenwood (SHA-WI and SHA-WIA); concentration of glucose in % of theoretical plotted against hydrolysis time in h.

Table 6. Effect of Time of Steam Treatment at 240°C, without Explosion, on Recovery of Steam-Heated Aspenwood (SHA); and, after Water Washing, on Recovery of Water-Soluble Fraction (WS) and Water-Insoluble Fraction (SHA-WI); and, after Alkali Washing, on Recovery of Alkali-Insoluble Fraction (SHA-WIA)

Treatment Time (s)	Recovery (% of original wood)			
	Total SHA	WS	SHA-WI	SHA-WIA
20	99.7	23.9	75.8	60.8
40	97.8	23.5	74.3	55.6
80	93.2	23.3	69.9	50.5
120	88.6	19.0	69.6	46.2
180	82.5	11.0	71.6	45.6

Table 7. Effect of Time of Steaming at 240°C, without Explosion, on Composition of the Water-Insoluble (SHA-WI) and Alkali-Insoluble (SHA-WIA) Fractions

Sample	Steaming time (s)	Analysis of sample (% of sample)[1]					
		Apparent Klason lignin[2]	Acid-soluble lignin	By HPLC		By Anthrone	Hexosan minus glucan
				Glucan[3]	Xylan	Hexosan	
SHA-WI	20	23.0	2.3	57.4	12.1	60.8	3.4
	40	25.6	2.0	60.1	8.8	62.3	2.2
	80	28.9	1.8	62.5	3.0	63.4	0.9
	120	30.7	1.9	58.6	1.4	62.9	4.3
	180	33.6	2.4	56.1	<1.0	60.4	4.3
SHA-WIA	20	15.5	1.7	66.6	9.3	72.9	6.3
	40	12.6	1.3	78.0	6.8	79.9	1.9
	80	8.4	0.6	84.4	1.7	86.6	2.2
	120	3.5	0.4	90.1	1.0	92.5	2.4
	180	2.1	0.3	91.9	<1.0	91.8	-0.1

[1] To convert to original-wood basis, multiply by apropriate recovery values listed in Table 6

[2] Apparent Klason lignin = true Klason lignin plus pseudolignin

[3] Glucan calculated from glucose and cellobiose

No systematic variation of enzyme loading was made in this study, in the absence of acidic catalysts. As described later in this report, however, such a study was made with the substrates obtained from SO_2-catalyzed steam treatments. Curves of glucose yield vs enzyme loading were obtained after 6, 12, 24 and 48 h for both SO_2-SHA-WI and SO_2-SHA-WIA. Although these curves were obtained at much lower enzyme loadings, and with a different enzyme preparation (Novozyme-Celluclast instead of E58), they were nevertheless used to adjust the hydrolysis yields from the SHA-WI substrates to the lower enzyme loadings of the corresponding SHA-WIA. Details of the method used are given in Appendix 2. A comparison is accordingly made in Table 8, of the glucose yields obtained by experiment from SHA-WI and SHA-WIA (at 35 FPU of E58 enzyme per gram of SHA-WI or SHA-WIA), with the calculated yields to be expected from SHA-WI if the enzyme loading were reduced to that of the corresponding SHA-WIA, on a per-gram-of-cellulose basis.

The virtually identical glucose yields, in the two right hand columns of Table 8 after 8, 24 and 48h of hydrolysis, indicate that, at equal enzyme loadings (per gram of cellulose), hydrolysis rates of SHA-WIA and SHA-WI are equal. After the longer time of 72h, the glucose yield from SHA-WIA was actually greater than that from SHA-WI, the hydrolysis curve of the latter having levelled.

The data of Table 8 were obtained from the 80s/240°C steam treatment. Similar calculations for the other steaming times of

Table 8. Comparison of Glucose Yields Obtained on Enzymatic Hydrolysis of SHA-WI and of SHA-WIA, with 35 FPU of E58 per Gram of Total Substrate; with the Yields Expected from SGA-WI if the Enzyme Loading Were to be Reduced to that of SHA-WIA if Both Loadings are Expressed per Gram of Cellulose

Hydrolysis time (h)	Glucose yield (% of theoretical)		
	By experiment		By calculation
	SHA-WI[1]	SHA-WIA[1]	SHA-WI[2]
8	49	38	38
24	76	60	59
48	85	72	72
72	86	85	76

[1] Data for 80s/240°C curves of Fig. 14. Enzyme loading 35 FPU/gram of total substrate

[2] Data obtained by calculation, using method described in Appendix 2, to estimate what glucose yield would have been obtained if the enzyme loading of SHA-WI had been reduced to that of SHA-WIA when both loadings are expressed per gram of cellulose

Fig. 14 (with few exceptions) showed much poorer or virtually no agreement. The virtually identical glucose yields obtained with the 80s/240°C materials may have resulted from the fact that these materials SHA-WI and SHA-WIA had cellulose and lignin contents very similar to those of the SO_2-SHA-WI and SO_2-SHA-WIA. Hydrolysis results from these latter two materials were used in preparing the calibration curves of glucose yield vs enzyme loading used in the calculations.

Fig. 14, without adjusting for enzyme loading, shows that the extending of steam-treatment times to longer than 80s, substantially improved hydrolysis with E58. For example, an increase in steaming time from 80s to 180s increased the hydrolysis yield from SHA-WI (with E58), from 74% to 91% of theoretical after 24h, and from 85% to virtually quantitative after 48h. Even when these yields are recalculated, so as to be based on the theoretical yields from the cellulose of the original wood, the yields after 180s of steaming are about 17% greater (after 24h), and 14% greater (after 48h), then the corresponding yields after 80s, (these increases being expressed as percent of the yields after 80s).

These increases as a result of longer steaming (without the use of peroxide) are somewhat greater than those observed in this laboratory under earlier contracts, and are greater than can be accounted for by decreasing cellulose contents and resulting increased enzyme loading per gram of cellulose. The difference may relate to the fact that in this more recent work the substrates were not autoclaved after enzymatic hydrolysis. Also,

they were not stored frozen. Both autoclaving, and storing frozen, have been found to have an adverse effect on enzymatic hydrolysis. The increased weight of cellulose converted to glucose, after steaming for 180s rather than for 80s, is, however, roughly balanced by an approximately equal or slightly greater weight loss of pentosan from the water-soluble fraction.

Comparison of the course of enzymatic hydrolysis for SHA-WIA (80s/240^{o}C) with E58 in Fig. 14, with that of the same material in Fig. 11, shows the latter to have been slower and less complete. This difference was due to the lower enzyme loading (25 IU of filter paper activity per gram of substrate in Fig. 11 compared with 35 IU in Fig. 14). The same batch of E58, suitably diluted, was used in both cases.

It can be concluded that when long steaming times and high loadings of a "good" enzyme preparation were used, high yields of glucose (approaching theoretical) were obtained even without added catalyst and without secondary peroxide treatment. When the steaming time was reduced to the extent that the glucose yield fell off substantially, then substantial improvements were obtainable with a secondary peroxide treatment. When, on the other hand, the steaming was adequate, but the enzyme loading was reduced to the point where the glucose yield fell off, then a secondary peroxide treatment caused an even greater improvement. Enzymatic hydrolysis was then faster and more complete.

The effects of concentration of hydrogen peroxide, and of time of reaction, were examined. It was found that the concentration

could be reduced to as low as 0.4%, before significantly reducing the effectiveness of the treatment. Also, the time could be reduced to 2 hours.

Wet Milling

Mechanical treatments, such as ball milling, have long been known to enhance subsequent enzymatic hydrolysis. Wood, however, is a tough material, and excessive energy requirements make such treatments uneconomical, if a substantial improvement in hydrolysis is required. After steam treatment, however, wood becomes much more friable and easy to grind. The use of ball milling as a "follow-up" treatment to a steam treatment was therefore examined.

Aspenwood which had been heated in steam at 240°C for 80 seconds, and exploded, was selected for this purpose because after 80 seconds much of the beneficial effect of steam treatment had already been reached. Three different relatively short times of milling were used with the wet water-washed steam-exploded wood. The moist milled products were then recovered on a suction filter. Details of the treatments are shown in Table 9, together with the very minor changes in composition. The results of enzymatic hydrolysis with E58 are presented in Table 10 and in Fig. 15.

Fig. 15 shows that milling after steam treatment also substantially increased both the rate and extent of enzymatic hydrolysis, with the improvement increasing with the time of milling. Six hours of milling increased the glucose yield, after

Table 9. Effect of Steam-Heating (240°C, 80s, With Explosion), Water-Washing, and Wet-Milling on Chemical Composition of Aspenwood

	Equivalent dry, ash-free[2] weight (g)	Composition[1] sample basis (%)		original wood basis (%)	
		P[3]	L[4]	P[3]	L[4]
Aspenwood	100	18.4	24.4	18.4	24.4
↓ steam treatment 240°C, 80s with explosion					
steam-exploded wood					
↓ water wash					
water-washed, steam-exploded wood	71.6	2.2	29.4	1.5	21.1
↓ wet mill, and filter					
Filter cake					
Time of milling (h)					
1.5	71.8	2.0	28.8	1.4	20.6
3.0	71.4	2.1	28.5	1.5	20.4
6.0	71.2	2.0	30.0	1.4	21.4

1 Composition on an oven-dry ash-free basis and, in the case of the lignin analysis, corrected for ash in the lignin

2 ash, abraded from ball-mill jars during wet milling

3 P - pentosan

4 L - total apparent lignin (i.e. true Klason lignin, acid-soluble lignin and pseudolignin)

Table 10. Effect of Wet Milling of Water-Washed Steam-Heated Aspenwood (240°C, 80s, With Explosion) on Subsequent Enzymatic Hydrolysis

Substrate	Concentration	Total Reducing Sugars (mg/mL)			Glucose (mg/mL)		
		6h	24h	48h	6h	24h	48h
Water-washed steam-exploded wood	20	8.0	10.4	10.6	7.9	10.4	11.3
	50	13.8	24.5	28.0	14.6	24.8	26.8
Steam-exploded wood, washed and ball-milled (1.5h)	20	8.4	10.7	11.2	8.3	11.3	11.9
	50	17.0	26.3	29.7	18.4	29.0	28.7
(3.0h)	20	8.8	11.5	11.8	9.5	11.7	13.0
	50	18.3	26.4	30.2	19.4	29.0	29.6
(6.0h)	20	10.5	12.1	12.8	10.6	12.9	13.8
	50	18.8	28.9	30.1	20.7	31.3	30.1

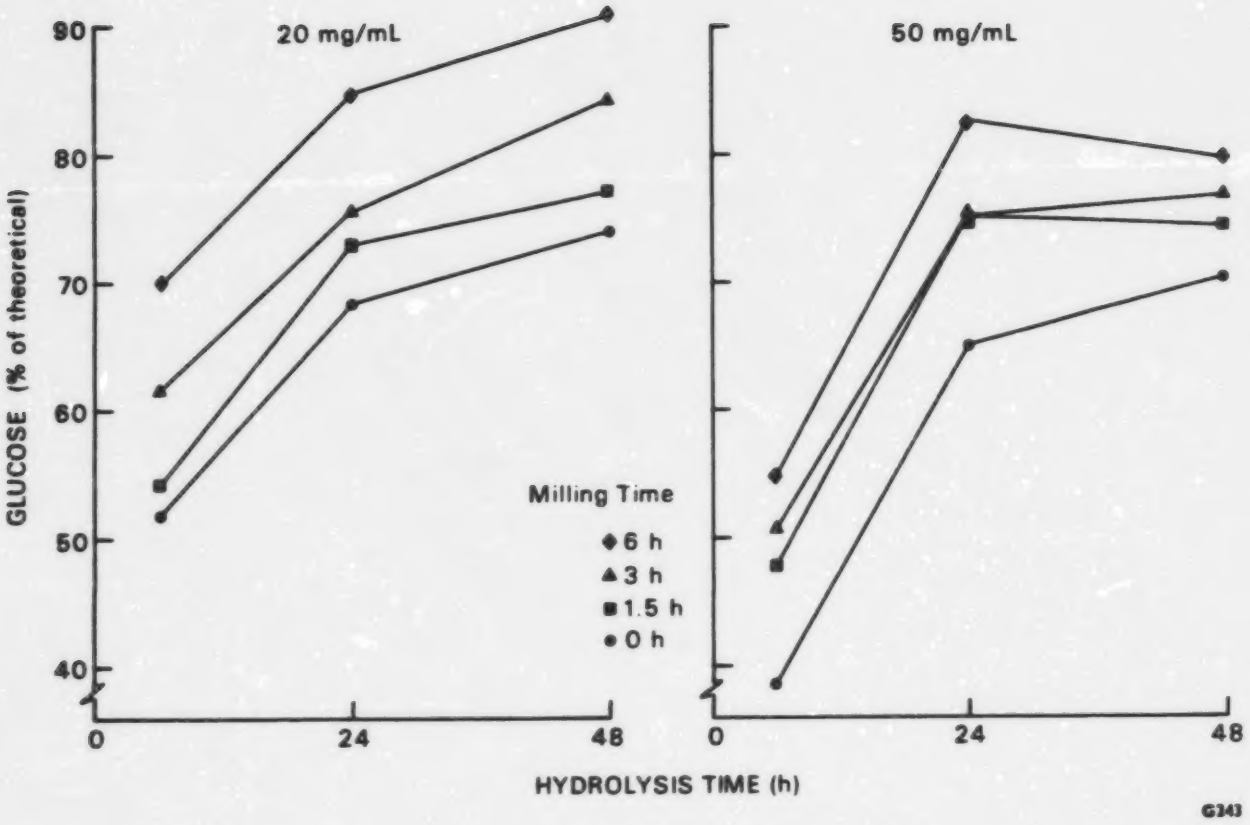


Figure 15. Effect of wet milling on enzymatic hydrolysis of water-washed steam-heated aspenwood (240°C, 80s, with explosion)

48 hours of enzymatic hydrolysis, from 74 to 91% of theoretical. No alkali extraction had been used, and no effort had been made to attain optimum milling conditions. Similar increases would probably result from the milling of wood steam-treated at 190°C.

Acid Impregnation

One well established way of minimizing pentosan destruction is, of course, to add an acidic catalyst. By this means, the solubilization of pentosan is also greatly accelerated, permitting, or even necessitating the use of relatively low temperatures. For example, Forintek (1), compared steam treatment at 240°C without acid, with treatment at 220°C after impregnation with sulfuric acid of concentration 0.2%. Maximum recovery of water-soluble pentosan in the acidic case was one-quarter greater than in that without added acid, and was obtained in one-third of the time, despite the 20°-lower temperature (Fig. 20 of Ref. 1). Clearly, acid impregnation aided in the separation of the hemicellulose component as water-soluble xylose and xylose oligomers. In an additional study at Forintek, not reported here in detail, a comparison was made of the following three treatments: 1) no added acid, 120s, 240°C; 2) 1.6% sulfur dioxide, 120s, 210°C; and 3) 0.2% sulfuric acid, 80s, 200°C. These treatments all resulted in approximately equal removal of pentosan after water washing (approximately 2% remaining). Approximately 13% of water-soluble pentosan was recovered in both the two acid cases, compared with only 9% in the no-acid case. Alkali extraction removed approximately 80% of the lignin in the no-acid and in the sulfur-dioxide cases, but only 52% in the

sulfuric-acid case. The lower yield in the sulfuric-acid case was related to a greater degree of condensation, as revealed by alkaline-nitrobenzene oxidation. Combined sulfur analyses suggested that solubilization of lignin in the sulfur-dioxide case may have resulted, in part, from sulfonation. These factors of lignin recovery, condensation and sulfonation, should be considered in relation to the end-use to which the by-product lignin is to be put. In addition, combined hydrolysis and fermentation gave significantly better yields of butanediol and ethanol in the two acid cases.

In a study of the lignin extracted from steam-exploded aspenwood (240^{o}C), without added catalyst, it was observed that, as the time of steam treatment was increased, the yields of vanillin and syringaldehyde to vanillin also decreased, indicating a different ratio of syringyl to guaiacyl groups in the lignin recovered. The amount of lignin extracted from the steam-exploded product also increased with time of steam treatment. It is not clear, therefore, to what extent the steam treatment itself caused increased condensation of the lignin, and to what extent the higher condensation merely reflects a different, more-difficulty-soluble fraction of lignin present in the original wood before steam treatment.

A comparison has been made of the effect of acid-impregnation, before steam treatment, on the fermentation inhibitors present in aqueous extracts of the steam-treated aspenwood. The inhibitors were extracted from the aqueous extracts by shaking repeatedly in

a separatory funnel with ethyl acetate. Gas chromatography of the ethyl acetate extracts was performed by Dr. Mackie of the New Zealand Forest Research Institute, using a technique developed for use with similar extracts obtained after conventional wood hydrolysis. The results showed most of the same inhibitors to be present, whether or not acid impregnation had been used, although the relative amounts of the individual compounds were different. Aqueous extracts after acid impregnation were at least as toxic to Zymomonas mobilis as were aqueous extracts without acid impregnation.

SO_2-Catalyzed Steam Treatment

In earlier work in 1984 (28), a preliminary study was made using SO_2-impregnated green (never-dried) aspenwood. This earlier work was done at Forintek in cooperation with Keith Mackie of the New Zealand FRI, who was on sabbatical at Forintek. Using 1.6% SO_2, (based on O.D. wood), and a steaming time of 120 seconds, a series of tre tments was made using steam temperatures of 201, 210, 220, and 227°C, the main purpose being to establish conditions for solubilization of approximately 90% of the pentosan. On the basis of this series, a steam temperature of 210°C was selected.

During the second year of the present contract, the same temperature of 210°C and impregnation with 1.6 SO_2 were used. Differences from the earlier work were: a faster loading of the SO_2-impregnated wood into the gun (as a result of replacement of the Parr-bomb lid, used in the earlier work, with a ball valve), and the use of the thin-walled canister in many of the

experiments. During the experiment described below, the opportunity was also taken to compare the different methods of analysis.

In the analysis of enzymatic hydrolysates, three methods have been used extensively, i.e. the Glucostat, YSI and DNS methods. Both the Glucostat and YSI (Yellow Springs Instrument Co.) methods, are based on the enzymatic formation of hydrogen peroxide from glucose, whereas the DNS (dinitrosalicylic acid) method is based on the reducing power of sugars. In the case of the Glucostat method, the amount of hydrogen peroxide formed is determined by the formation of a colored compound by reaction with o-dianisidine in the presence of a second enzyme (a peroxidase). In the YSI method the oxidase enzyme is immobilized within a membrane, and the hydrogen peroxide formed is measured in the YSI Model 27 by electrochemical oxidation at a platinum electrode. The Glucostat and YSI methods, because of the specificity of the oxidase enzyme involved, should measure only glucose. The DNS method should include all reducing sugars and possibly other reducing material that might be present.

The first series of treatments, described below as Series 1, was done to observe any possible difference resulting from different ratios of weight of wood charge to gun volume, as a consequence of using the canister. The 2-litre gun barrel normally holds a full charge of 200 equivalent dry weight of wood chips, whereas the full canister normally holds only 67g. Table 11 shows that after 120 s treatment in steam at 210^{o}C, only very slight

Table 11. Comparison of recovery and water-solubility of aspenwood impregnated with 1.6% SO_2 and heated for 120 s in 210°C steam in fully charged gun or in filled or half-filled canister.

(Series 1)

	SO_2 (% on O.D. wood)	Wood charge dry eq.wt. (g)	With or without canister	With or without explosion	Recovery from gun (% of input wood)		
					Water solubles	Water insolubles	Total
1	1.60	200	no canister	exploded	31.3	65.7	96.9
2	1.64	67	with canister	not exploded	32.9	65.2	98.1
3(a)	1.60	33.5	with canister	not exploded	33.1	64.4	97.5
3(b)	1.64	33.5	with canister	not exploded			

differences in the extent of solubilization of SO_2-impregnated aspenwood were obtained from a full-gun charge, a full-canister charge and a half-canister charge.

The effect of varying the time of steaming of full-canister charges is shown in Tables 12 and 13, for a second series of treatments of SO_2-impregnated wood (Series 2). Although SO_2 catalyzed the desirable process of hydrolysis, recovery of the steamed product (SHA) decreased with increasing steaming time and after 120 s at 210°C was approximately equal to that after the same time at 240°C without SO_2 (Tables 1 and 6). After 120 s, however weight was lost more slowly than in the uncatalyzed case, reflecting less destruction of xylose. The amounts of both monomeric xylose and glucose, found in the water-soluble fraction by the two HPLC columns, (Table 12) are in excellent agreement. Monomeric xylose passed through a maximum at 120 s, whereas glucose continued to increase at least to 200 s, at which time it represented approximately 15% of the cellulose or other glucan of the original wood. The amount of acetic acid increased with steaming time, until at 200 s, it was equivalent to 4.1% acetyl in the original input wood, i.e. to essentially all of the original wood acetyl.

HPLC chromatograms of water solubles are presented in Fig. 16, 17, 18 and 19 after an 80 s, 240°C treatment without added SO_2, and after SO_2-catalyzed treatments for 40, 120 and 200 s at 210°C, respectively. These chromatograms show, in the no-SO_2 case, a relatively large amount of hemicellulose oligomers which, in the SO_2 cases, are much reduced even after only 40 s, and are

Table 12. Effect of time of steaming of aspenwood[1] impregnated with SO_2, in 210°C steam in canister[2], on recovery from gun and on solubilization of xylan and glucan[3]

(Series 2)

	SO_2 (% on O.D. wood)	Steaming time (s)	Recovery of SHA (% of original wood)	Moisture content (%, wet basis)	Xylose, glucose and acetic acid in water solubles calculated as xylan, glucan and acetyl[4] and expressed as % of input wood				
					XYLOSE		GLUCOSE		ACETIC ACID
					P. column	H. column	P.column	H.column	H. column
1.	1.61	40	94.4	69.3	9.7	-[5]	2.3	3.7	1.5
2.	1.63	80	92.6	69.5	12.4	-[6]	3.6	2.8	2.3
3.	1.67	120	89.7	72.1	13.1	13.8	4.9	4.2	2.8
4.	1.61	160	89.6	70.9	11.5	11.3	5.6	4.8	3.0
5.	1.61	200	89.2	74.2	11.4	11.8	6.8	6.9	4.1

1) small green never-dried chips
2) canister filled with chips, equivalent dry weight 67 g
3) glucan, including cellulose
4) acetyl group (CH_3CO)
5) No meaningful value can be given for xylose from the H column for sample 1, because of interference with another peak. Failure to notice this shoulder on the xylose peak would lead to erroneously high value of 15.9.
6) As in footnote 5, the xylose peak displayed an obvious shoulder. Improper inclusion of this shoulder would lead to an incorrect high value of 14.4.

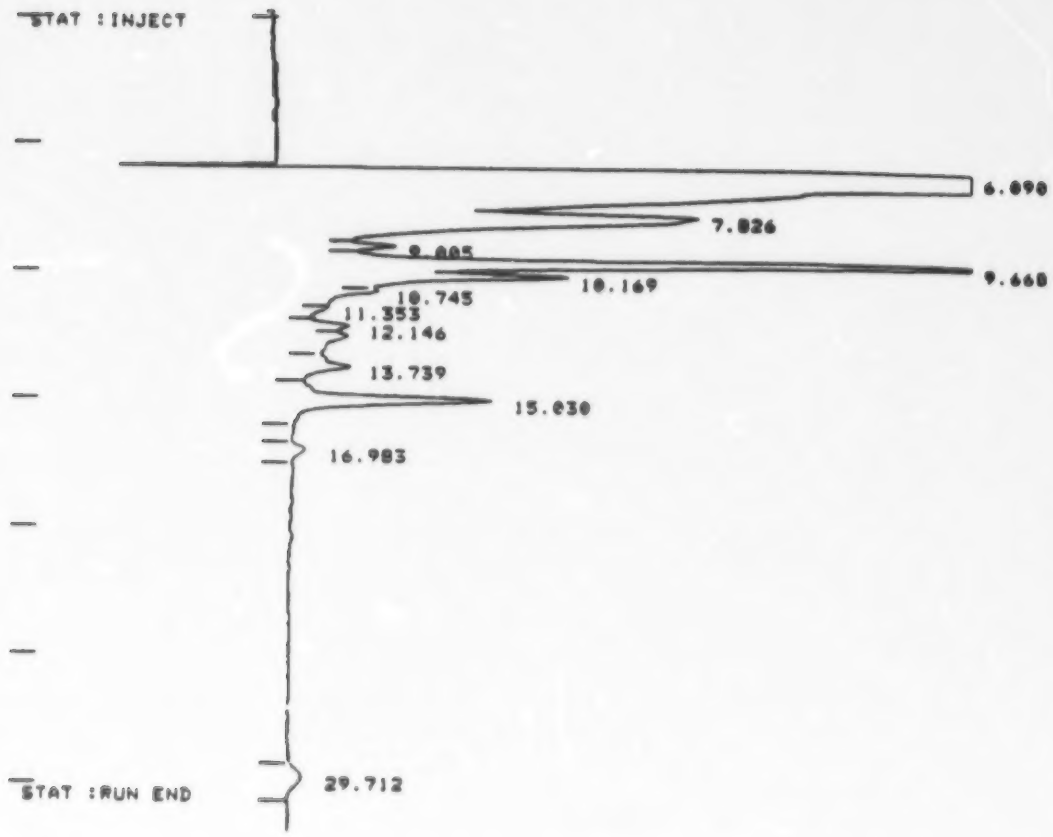
Table 13. Recovery and analysis of water insolubles (WI) from Series 1 and Series 2

	Steaming time (s)	Yield of W.I. (% of input wood)	Analysis of water-insolubles				
			Lignin (% of W.I.)			Carbohydrate (% of W.I.)	
			Apparent Klason lignin	Acid-soluble lignin	Total apparent lignin	Hexosan (Anthrone)	Glucan (HPLC)
Series 1							
1	120	65.6	28.4	1.5	29.9	65.8	59.6
2	120	65.2	29.1	1.9	31.0	65.3	60.5
3	120	64.4	29.7	1.9	31.6	64.7	58.6
Series 2							
1	40	64.5	28.0	1.4	29.4	66.1	61.7
2	80	62.5	29.9	1.6	31.5	66.5	61.0
3	120	61.4	30.0	1.9	31.9	64.7	60.0
4	160	59.9	30.1	1.9	32.0	63.7	59.2
5	200	59.6	31.1	2.0	33.0	62.4	58.4
Old SO_2 treatment[1] with Mackie	120	65.2	29.4	1.6	31.0	64.8	59.7
Old peroxide treatment[2]	-	-	10.7	0.9	11.6	81.6	78.3
Original input wood[3]	0	-	21.3	2.5	23.9	47.7	44.2

1) 1984 sample, 120 s, 210°C, 1.6% SO_2, (comparable with Series 1 sample 1) stored frozen.

2) Steam heated aspenwood (25 min, 190°C) water and alkali washed, peroxide treated (prepared as Pulp # 1 of Table 4), stored frozen apprimately 1 year.

3) Analysis of whole unextracted aspenwood, before steam treatment.



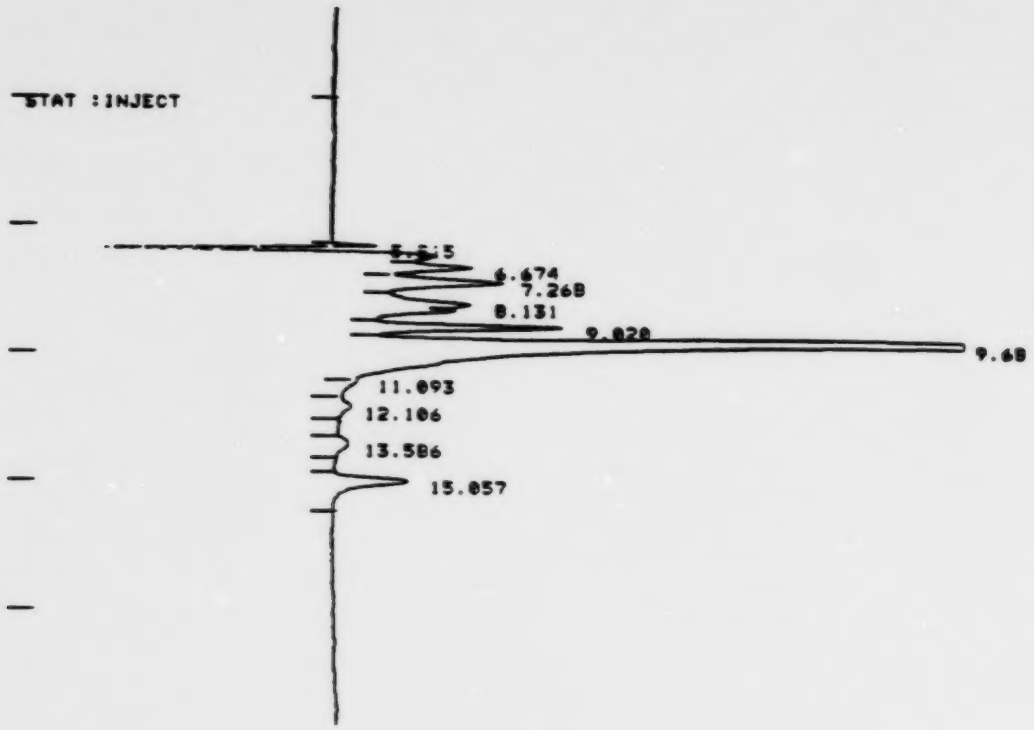
TITLE: 17:02 13 DEC 86

CHANNEL NO: 2 SAMPLE: 80/240 METHOD: LCARBH

PEAK NO	PEAK NAME	RESULT	TIME (MIN)	AREA COUNTS	BEP CODE
1		0.0000	6.090	92028	BV
2		0.0000	7.826	29690	VV
3	GLUCOSE	0.4278	9.005	5450	VV
4	XYLOSE	1.8170	9.668	23193	VV
5		0.0000	10.169	11313	VV
6		0.0000	10.745	6317	VV
7		0.0000	11.353	3850	VV
8		0.0000	12.146	3382	VV
9		0.0000	12.531	5400	VV
10		0.0000	13.739	4481	VV
11	ACETIC	1.1565	15.030	4975	VB
12		0.0000	16.983	383	BB
13		0.0000	29.712	815	BB
TOTALS:		3.4013		192559	

DIVISOR: 1.00000 MULTIPLIER: 1.00000

Fig. 16. HPLC chromatogram of water-soluble fraction of 80s/240°C steamed aspenwood-no SO_2



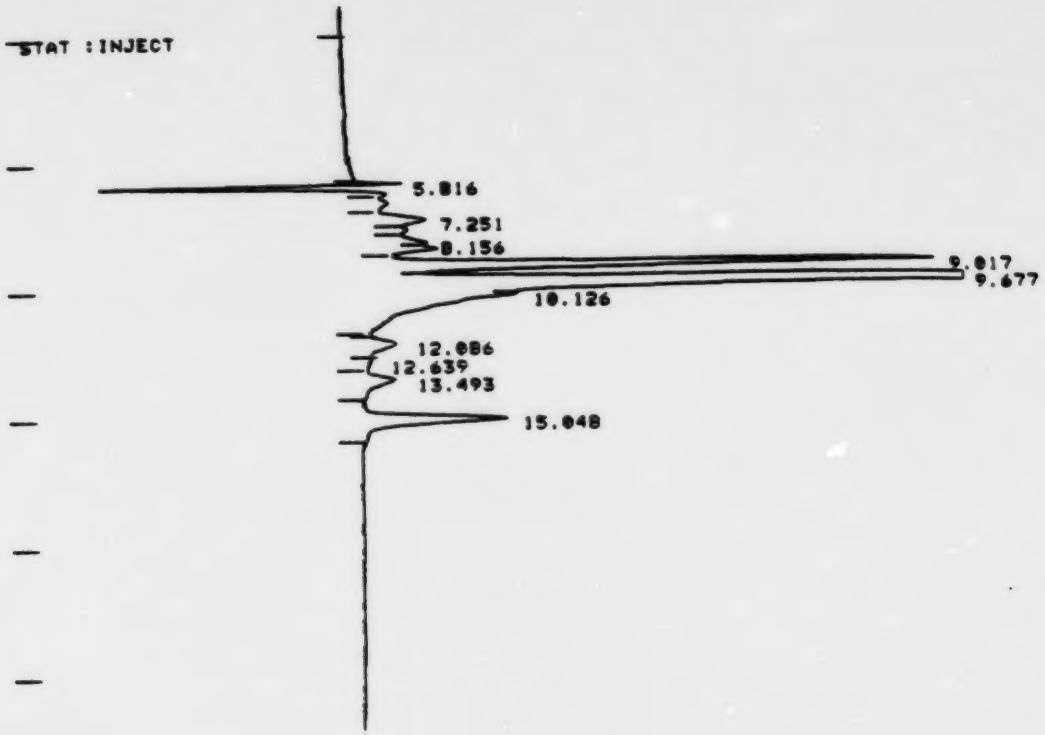
TITLE: 11:10 17 DEC 86

CHANNEL NO: 2 SAMPLE: 40/210/SO2 METHOD: LCARBH

PEAK NO	PEAK NAME	RESULT	TIME (MIN)	AREA COUNTS	SEP CODE
1		0.0000	5.815	1131	BV
2		0.0000	6.198	7919	VV
3		0.0000	6.674	9692	VV
4	CELLOBIOSE	0.9697	7.268	12778	VV
5		0.0000	8.131	9591	VV
6		0.0000	8.276	6355	VV
7	GLUCOSE	0.6994	9.020	8909	VV
8	XYLOSE	3.1728	9.684	40433	VV
9		0.0000	11.093	2061	VV
10		0.0000	12.106	1166	VB
11		0.0000	13.586	399	BB
12	ACETIC	0.3500	15.057	2111	BB
TOTALS:		5.1919		102545	

DIVISOR: 1.00000 MULTIPLIER: 1.00000

Fig. 17. HPLC chromatogram of water-soluble fraction of 40s/210°C steamed aspenwood with 1.6 percent SO_2



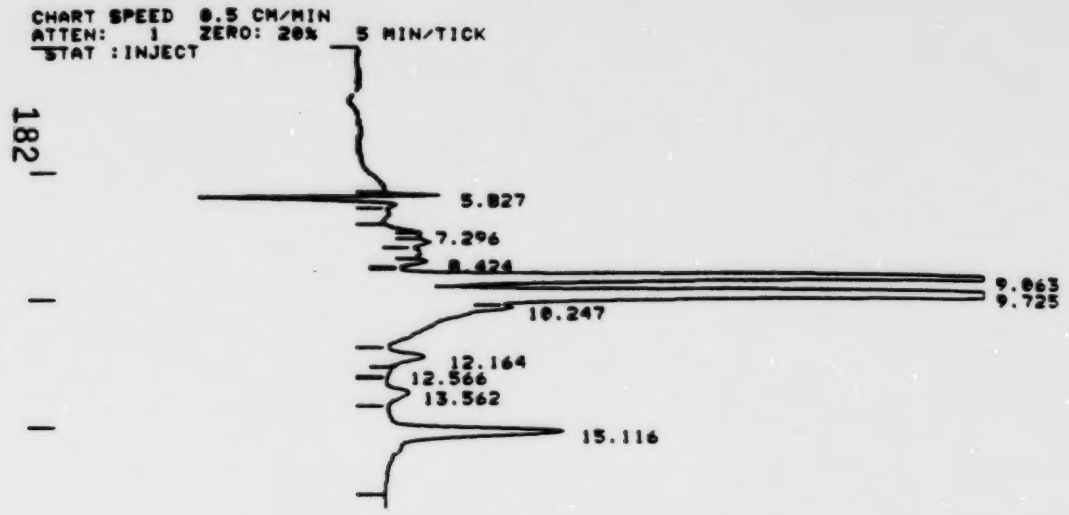
TITLE: 12:25 17 DEC 86

CHANNEL NO: 2 SAMPLE: 120/210/SO2 METHOD: LCARBH

PEAK NO	PEAK NAME	RESULT	TIME (MIN)	AREA COUNTS	SEP CODE
1		0.0000	5.816	1370	BV
2		0.0000	6.220	1994	VB
3	CELLOBIOSE	0.0899	7.251	1185	BV
4		0.0000	7.633	616	VV
5		0.0000	8.156	1108	VV
6		0.0000	8.396	1201	VV
7	GLUCOSE	0.8027	9.017	10226	VV
8	XYLOSE	2.4681	9.677	31453	VV
9		0.0000	10.126	5364	VB
10		0.0000	12.086	820	BV
11		0.0000	12.639	106	VV
12		0.0000	13.493	913	VV
13	ACETIC	0.6068	15.048	3660	VB
TOTALS:		3.9675		60816	

DIVISOR: 1.00000 MULTIPLIER: 1.00000

Fig. 18. HPLC chromatogram of water-soluble fraction of 120s/210°C steamed aspenwood with 1.6 percent SO_2



TITLE: 13:44 23 DEC 86

CHANNEL NO: 2 SAMPLE: 200/210/SO2 METHOD: LCARBH

PEAK NO	PEAK NAME	RESULT	TIME (MIN)	AREA COUNTS	BEP CODE
1		0.0000	5.827	1170	BV
2		0.0000	6.180	1427	VB
3		0.0000	7.677	210	BB
4		0.0000	8.424	209	BB
5	GLUCOSE	1.2482	9.063	15901	BV
6	XYLOSE	2.3389	9.725	29806	VV
7		0.0000	10.247	5259	VV
8		0.0000	12.164	1004	VV
9		0.0000	13.562	780	VV
10	ACETIC	1.0117	15.116	6102	VB
TOTALS:		4.5988		61868	

DIVISOR: 1.00000 MULTIPLIER: 1.00000

Fig.19. HPLC chromatogram of water-soluble fraction of 200s/210°C steamed aspenwood with 1.6 percent SO_2

replaced by much more monomeric xylose. A pronounced increase in glucose with increasing time, in the SO_2 case is also obvious, as is the increase in acetic acid.

Table 13 shows the analyses and yields of the water-washed products of Series 1 and 2, and also includes analyses (done at the same time) of an old SO_2-treated sample, stored in a deep freeze, from the 1984 study with Mackie. An old peroxide-treated pulp (Pulp #1, Table 4) stored, frozen, for 1 year, was also re-analyzed and included.

Comparison of the glucan analyses (as calculated from the glucose and cellobiose values obtained by HPLC), with the hexosan analyses (obtained by the anthrone method), show that the hexosan values were higher by an average of 4.8 percentage points. Both show the same trend downward, with increasing treatment time in Series 2. This downward trend is the result of cellulose solubilization. Little, if any, glucose destruction occurred. If, in fact, the hexosan values are converted to an original-wood basis by multiplication by the listed yield values, and if to the results thus calculated are added the glucan equivalents of the glucose found in the corresponding water-soluble fractions reported in Table 12 (P column), an almost constant total value is obtained. The totals all lie between 45.2 and 43.8% with little trend downward, showing that, although significant solubilization of cellulose occurred, virtually no destruction of the resulting glucose followed. The slightly higher hexosan content of the original wood (47.7%) included mannose and

galactose (virtually absent from the steamed-and-washed products), which should account for between 2 and 4% of this 47.7% making the agreement very good.

The differences, however, between the hexosan and glucan values for all of the samples listed, except in the case of the original wood, must be considered as discrepancies between the two analytical methods. In calculating the theoretical yields of glucose to be expected on enzymatic hydrolysis, therefore, different values are obtained depending on whether the anthrone or HPLC values for the substrate are used. Additional theoretical yields can also be calculated, based on the composition of the original input wood and on the yield of the water-washed steamed product. These additional theoretical yields can also be corrected for the glucose found in the water solubles and therefore not available for enzymatic hydrolysis. The calculated theoretical yields are listed in Table 14. A good agreement can be seen between the theoretical yields based on the anthrone hexosan analyses of SHA-WI, and the theoretical yields based on the HPLC glucan analysis of the input wood, corrected for glucose already removed in the water-washings.

The actual concentrations of glucose, cellobiose and total reducing sugars obtained on enzymatic hydrolysis at a 2% consistency, are listed in Table 15, as determined by YSI, Glucostat, HPLC and DNS. The differences between the three glucose values (YSI, Glucostat and HPLC) obtained for each sample, represent discrepancies between the three analytical methods, and not differences in the enzymatic hydrolysis. The

Table 14. Comparison of calculated possible theoretical yields of glucose in enzymatic hydrolysates of water-washed steam-treated aspenwood (SHA-WI) of Series 1 and Series 2, as calculated from the composition of the water-washed steamed samples, and from the composition of the original input wood

	Steaming time (s)	Based on composition of SHA-WI determined by:		Based on composition of input wood determined by HPLC	
		Anthrone hexosan (mg/mL)	HPLC glucan (mg/mL)	disregarding loss of glucan in WS[1] (mg/mL)	reduced by amount of glucan in WS[1] (mg/mL)
Series 1					
1	120	14.6 [4]	13.2	14.9	-
2	120	14.5	13.5	15.1	-
3	120	14.4	13.0	15.2	-
Series 2					
1	40	14.7	13.7	15.2	14.4
2	80	14.8	13.6	15.7	14.4
3	120	14.4	13.3	16.0	14.2
4	160	14.1	13.1	16.4	14.3
5	200	13.9	13.0	16.5	13.9
Old SO_2 treatment[2]	120	14.4	13.3	14.9	-
Old peroxide treatment[3]	-	18.1	17.4	-	-

1) glucose present in water solubles (Table 12)

2) 3) Materials identified in Footnotes 1 and 2 of Table 13

4) Example calculation: (Series 1 - #1) Enzymatic hydrolysis - 2% slurry of SHA-WI i.e. 20 mg/mL
Hexosan(anthrone) content, from Table 13 - 65.8%
Theoretical glucose conc. = $20 \times \frac{65.8}{100} \times \frac{180}{162}$ = 14.6 mg/mL

Table 15. Analysis of enzymatic hydrolysates[1] of water-washed steam-treated wood

Hydrolysis time (h)		Steaming time (s)	Glucose (mg/mL) by: YSI	Glucostat	HPLC	Cellobiose by HPLC (mg/mL)	Total reducing sugars by DNS (mg/mL)
8	Series 1						
	1	120	6.09	6.29	6.32	1.14	7.66
	2	120	6.32	6.83	6.56	1.34	7.91
	3	120	6.09	6.38	6.64	1.50	7.64
	Series 2						
	1	40	6.84	6.84	7.36	2.11	8.53
	2	80	7.36	7.77	7.75	2.43	9.68
	3	120	7.52	7.69	7.76	2.26	9.49
	4	160	7.84	7.89	8.02	2.08	9.79
	5	200	8.12	8.05	8.51	2.45	9.95
	Old SO_2	120	3.90	3.86	4.23	0.55	4.85
	Old Peroxide	-	3.81	3.96	4.31	0.57	4.92
24	Series 1						
	1	120	10.48	9.91	10.53	1.12	11.21
	2	120	11.28	11.10	11.57	1.41	12.92
	3	120	11.12	10.52	11.17	0.80	12.00
	Series 2						
	1	40	10.99	10.44	11.25	0.66	12.21
	2	80	12.52	11.76	12.68	1.74	13.14
	3	120	12.64	12.02	12.79	0.73	13.59
	4	160	13.59	12.69	12.93	0.73	14.39
	5	200	13.24	12.77	12.89	1.09	14.41
	Old SO_2	120	6.72	6.73	7.08	0.41	7.52
	Old Peroxide	-	6.79	7.02	7.09	0.37	8.09

Table 15. Analysis of enzymatic hydrolysates[1] of water-washed steam-treated wood Cont'd

Hydrolysis time (h)		Steaming time (s)	Glucose (mg/mL) by: YSI	Glucostat	HPLC	Cellobiose by HPLC (mg/mL)	Total reducing sugars by DNS (mg/mL)
48	Series 1						
	1	120	12.59	12.72	12.28	0.39	13.35
	2	120	13.71	13.93	13.36	0.27	14.68
	3	120	13.59	13.47	13.16	0.56	14.53
	Series 2						
	1	40	13.07	13.60	12.90	0.57	14.16
	2	80	14.08	14.43	14.00	0.28	15.02
	3	120	14.02	14.60	13.71	0.37	14.98
	4	160	14.45	14.93	14.51	0.45	15.22
	5	200	15.12	14.88	14.36	0.37	15.78
	Old SO_2	120	8.55	9.19	8.50	0.32	9.39
	Old Peroxide	-	9.04	9.37	8.94	0.28	10.67
72	Series 1						
	1	120	13.68	14.40	13.25	0.33	15.06
	2	120	14.65	14.53	13.77	0.27	15.22
	3	120	14.64	14.91	14.59	0.31	15.66
	Series 2						
	1	40	13.97	14.98	14.29	0.30	15.58
	2	80	14.62	15.67	15.05	0.28	16.37
	3	120	15.03	15.85	14.70	0.15	15.53
	4	160	15.21	15.86	14.72	0.19	16.02
	5	200	15.22	16.23	15.06	0.29	16.30
	Old SO_2	120	9.70	10.68	9.80	0.24	10.52
	Old Peroxide	-	10.56	11.05	11.08	0.30	12.04
96	Series 1						
	1	120	14.27	14.48	13.63	0.22	14.66
	2	120	15.17	15.23	13.96	0.22	15.27
	3	120	14.90	14.82	13.81	0.15	15.07

Table 15. Analysis of enzymatic hydrolysates[1] of water-washed steam-treated wood Cont'd

Hydrolysis time (h)	Steaming time (s)	Glucose (mg/mL) by: YSI	Glucostat	HPLC	Cellobiose by HPLC (mg/mL)	Total reducing sugars by DNS (mg/mL)
Series 2						
1	40	14.76	15.51	14.31	0.27	15.00
2	80	15.28	15.38	14.89	0.31	15.47
3	120	15.09	15.45	15.04	0.18	15.73
4	160	15.74	15.97	13.26	0.16	15.76
5	200	15.97	15.91	14.59	0.17	16.17
Old SO_2	120	10.82	11.12	9.70	0.25	11.37
Old Peroxide	-	11.60	11.56	11.31	0.36	13.15

1) Enzymatic hydrolysis: total volume 200 mL
dry equivalent weight of substrate 4.00 g (i.e. 2% slurry)
30 FPU enzyme (E58) per gram of substrate
aliquots, of 3.0 mL each, removed at 8, 24, 48, 72 and 96h for analysis
values listed are adjusted to correct for aliquots already removed, as described in Appendix 3.

three aliquots were taken from the same hydrolysis mixture in each case. Also, all enzymatic hydrolyses reported in Table 15 were done with the same freeze-dried enzyme preparation.

It can be seen that, in general, after longer hydrolysis time, the Glucostat method gave significantly higher values than did the HPLC. This discrepancy has been repeatedly observed in other experiments not reported here. Despite experiments done specifically to determine the cause, the reason is still not known. There is no apparent problem in applying HPLC, with the P column, to enzymatic hydrolysates. No neutralization is required and no hydrolysis loss factor is needed.

The data of Table 15 are expressed in Table 16 as percent of theoretical, using the anthrone hexosan values of Table 14 as theoretical. It can be seen that in several cases, especially after longer hydrolysis times, yields above 100% are indicated. Although these excessively high values are particularly evident in the Glucostat data, they are also shown in lesser degree by the HPLC.

The reason for the erroneously high hydrolysis yields in the HPLC case is not known. Sampling of woody materials, when the moisture content is well above the fiber saturation point, is always a potential source of error. It is, however, unlikely that the high yields results from unrepresentative sampling of the wet substrates (M.C. approximately 80%, wet basis), of from partial drying during storage between M.C. determination and hydrolysis, because the yield trend in Series 2 is reasonably

Table 16. Comparison of yields of glucose plus glucose equivalent of cellobiose,[1] obtained on enzymatic hydrolysis of water-washed steam-treated wood (SHA-WI), as measured by YSI, Glucostat, and HPLC analysis of enzymatic hydrolysates, and expressed as percent of theoretical glucose as calculated from analysis of SHA-WI[2]

Hydrolysis time (h)		Steaming time (s)	Yield of glucose + glucose equivalent of cellobiose (% of theoretical)[2]		
			YSI	Glucostat	HPLC
8	Series 1				
	1	120	49.8	51.2	51.4
	2	120	53.3	56.8	54.9
	3	120	53.4	55.4	57.2
	Series 2				
	1	40	61.7	61.7	65.2
	2	80	67.1	69.8	69.7
	3	120	68.8	70.0	70.5
	4	160	70.9	71.3	72.2
	5	200	77.2	76.7	80.0
	Old SO_2	120	31.1	30.8	33.4
	Old Peroxide	-	24.3	25.1	27.1
24	Series 1				
	1	120	79.7	75.8	80.0
	2	120	88.0	86.7	90.0
	3	120	83.3	79.1	83.6
	Series 2				
	1	40	79.6	75.8	81.3
	2	80	97.1	91.9	98.1
	3	120	93.3	88.9	94.3
	4	160	101.5	95.2	96.8
	5	200	103.8	100.4	101.3
	Old SO_2	120	49.7	49.8	52.2
	Old Peroxide	-	39.6	40.9	41.2

Table 16. Comparison of yields of glucose plus glucose equivalent of cellobiose,[1] obtained on enzymatic hydrolysis of water-washed steam-treated wood (SHA-WI), as measured by YSI, Glucostat, and HPLC analysis of enzymatic hydrolysates, and expressed as percent of theoretical glucose as calculated from analysis of SHA-WI[2]

Cont'd

Hydrolysis time (h)	-	Steaming time (s)	Yield of glucose + glucose equivalent of cellobiose (% of theoretical)[2]		
			YSI	Glucostat	HPLC
48	Series 1				
	1	120	88.9	89.7	86.7
	2	120	96.5	98.0	94.1
	3	120	98.7	97.9	95.7
	Series 2				
	1	40	93.1	96.7	91.9
	2	80	97.2	99.6	96.7
	3	120	100.2	104.3	98.1
	4	160	105.5	108.9	105.9
	5	200	111.9	110.2	106.4
	Old SO_2	120	61.8	66.2	61.4
	Old peroxide	-	51.5	53.3	50.9
72	Series 1				
	1	120	95.9	100.8	92.9
	2	120	102.9	102.1	96.9
	3	120	104.2	106.1	103.8
	Series 2				
	1	40	97.3	104.2	99.5
	2	80	100.9	108.0	103.8
	3	120	105.6	111.3	103.3
	4	160	109.0	113.5	105.5
	5	200	112.0	119.3	110.8
	Old SO_2	120	69.2	76.0	69.9
	Old Peroxide	-	60.0	62.7	62.8
96	Series 1				
	1	120	99.1	100.6	94.7
	2	120	106.2	106.6	97.8
	3	120	104.8	104.3	97.2

Table 16. Comparison of yields of glucose plus glucose equivalent of cellobiose,[1] obtained on enzymatic hydrolysis of water-washed steam-treated wood (SHA-WI), as measured by YSI, Glucostat, and HPLC analysis of enzymatic hydrolysates, and expressed as percent of theoretical glucose as calculated from analysis of SHA-WI[2] Cont'd

Hydrolysis time (h)		Steaming time (s)	Yield of glucose + glucose equivalent of cellobiose (% of theoretical)[2] YSI	Glucostat	HPLC
96	Series 2				
	1	40	102.5	107.6	99.4
	2	80	105.5	106.2	102.9
	3	120	106.3	108.8	105.9
	4	160	112.5	114.1	94.9
	5	200	116.5	116.1	106.5
	Old SO_2	120	77.0	79.1	69.2
	Old peroxide	-	66.1	65.8	64.5

1) Glucose equivalent of cellobiose = weight of cellobiose x 1.0526

The factor 1.0526 is $\frac{2 \times \text{mol. wt. of glucose}}{\text{Mol. wt. of cellobiose}}$

Yields listed are the total yields of glucose which would have been obtained if the cellobiose (found by HPLC) had been obtained as glucose.

2) Values used for theoretical, are the values listed in Column 3 of Table 14, obtained from the analysis of the water-washed steam-heated wood (SHA-WI) by the anthrone method.

e.g. for sample 1 of Set 1 (Table 14) the theoretical glucose conc. listed is 14.6 mg/mL. After 8h the adjusted observed glucose conc. (Table 15) was 6.09 mg/mL as obtained by YSI and the cellobiose conc. was 1.14 mg/mL by HPLC

$$\text{Yield} = \frac{[6.09 + (1.0526 \times 1.14)]100}{14.6} = 49.8\% \text{ as listed in Table 16.}$$

smooth. They could have resulted from erroneously low theoretical values (Table 14), calculated from possibly erroneously low hexosan or glucan analyses of the substrates (Table 13). The HPLC-obtained glucan value of 44.2%, listed for the original aspenwood, does appear low when compared with the mean value of 47.6% obtained in the CPD-2 project with Forintek-supplied P. tremuloides wood. The high yield values, however, were based on the anthrone-derived hexosan analyses which were significantly higher. Further work on carbohydrate analysis, and the reasons for the errors are discussed later in this report.

A possibly very significant difference in yield on enzymatic hydrolysis is shown in Table 16, between the 120 s products in the present work, and the 120 s product from the earlier work with Mackie. All samples were enzymatically hydrolyzed at the same time, with the same freeze-dried batch of E58 enzyme. The old water-washed sample, however, which had been stored in the deep freeze since 1984, gave yields only one-half to two-thirds as great as did the fresh never-frozen products, even though, as shown in Table 13, the chemical compositions were very similar.

It is not clear whether this great difference in hydrolysis yield was caused by the storage in the deep freeze, or by the loss of some of the SO_2 in the early work during the longer time required to close the Parr bomb lid. In any case, no more than half of the SO_2 was lost even after the explosive decompression of the charge (28). Presumably much of what was lost escaped with the steam only during the explosion, i.e. after the

treatment had been completed. In the present work, all of the fresh SO_2 samples of Series 2 (from 40 s to 200 s) gave hydrolysis yields much higher than did the old 120 s SO_2 sample, suggesting that, even if a significant amount of SO_2 had been lost, a good yield would have been obtained.

A similar large decrease in enzymatic hydrolyzability also occurred, apparently as a result of storing a peroxide-treated sample in the deep freeze for approximately one year. This old sample (Table 16) had been treated with hydrogen peroxide by the same chemist at the same time, and by the same method as had Pulp #1 of Table 4. Table 5 and subsequent work had been obtained from Pulp #1 a year ago, immediately after it had been freshly prepared.

It is probable that the reduced yields, obtained on enzymatic hydrolysis of both the SO_2- and peroxide-treated samples, resulted from the storage. Air-drying of moist steamed wood has been shown to reduce the hydrolysis yield. It has also been observed that improper storage, even in tightly closed containers in the freezer compartments of refrigerators, can cause migration of virtually all of the moisture out of the steamed wood. The moisture moves towards the colder side of the container, leaving the wood as an apparently air-dry powder completely separated from a pile of ice crystals. In both the SO_2 and peroxide cases, however, there was no evidence of this type of "freezer drying".

Comparison of Uncatalyzed and SO_2-Catalyzed Treatments

The data of Tables 17 and 18, which were obtained without the use

Table 17 : Analysis of water-insoluble fractions (WI) of aspenwood treated in steam at 240°C without SO_2

Steaming time (s)	Apparent Klason lignin (%)	Acid-soluble lignin (%)	Total apparent lignin (%)	Hexosan (anthrone) (%)
20	23.0	2.3	25.3	60.8
40	25.6	2.0	27.6	62.3
80	28.9	1.8	30.7	63.4
120	30.7	1.9	32.6	62.9
180	33.6	2.4	36.0	60.4
Original wood	19.8	3.3	23.1	47.2

Table 18 . Yields of glucose obtained on enzymatic hydrolysis of water-washed steam-treated wood (SHA-WI) (no SO_2) (240°C) as measured by HPLC and expressed as percent of theoretical glucose as calculated from anthrone analysis of SHA-WI

Steaming time (s)	Yield[2] of glucose (% of theoretical)			
	8 h	24h	48h	72h
20	30.5	45.7	55.4	56.3
40	33.2	50.4	59.4	62.1
80	48.2	75.2	85.1	85.9
120	51.3	81.9	93.1	91.5
180	55.9	92.0	102.2	98.5

1) total substrate consistency: 20 mg/mL as in Table 13; 16 Table 16
enzyme: freeze-dried E58 from same batch as used in SO_2 experiments of Table 13; 16
enzyme loading: 30 FPU per gram of total substrate

2) yields are of glucose only, and do not include the glucose equivalent of the cellobiose. (The glucose/cellobiose relative amounts are shown in Table 12). 15 Table 15

of SO_2, are included here for comparison with the above SO_2 data (Tables 15 and 16). The E58 enzyme used was from the same batch in both cases and was used at the same loading (30 FPU/g substrate). Table 18 shows that, even without the SO_2, very good glucose yields were obtained after 48 hours of enzymatic hydrolysis, for aspenwood samples treated in 240°C steam for times of 80 s or longer. Comparison with Table 16 indicates that, with SO_2, and a similar treatment time, but in steam at the lower temperature of 210°C, comparable good yields were obtained in about half the time (i.e. 24 hours).

In addition to faster development of cellulose accessibility, the use of SO_2 results in less destruction of pentosan for the same degree of accessibility. Furthermore, in the SO_2 case, a much greater fraction of the solubilized xylan is present as monomeric xylose rather than as oligomers, as has been described above.

Incorporation of Sulfur in SO_2-Catalyzed Product

A potential disadvantage in the use of SO_2 is the possible incorporation of sulfur in the product, possibly as sulfonic acid groups in the lignin. Wayman has reported no substitution. Mackie and Brownell (28) have reported residual sulfur in steam-exploded SO_2-impregnated aspenwood, even after prolonged washing which reduced the sulfur content from 0.42% in the unwashed steam-exploded product, to 0.08% in the washed product as determined by combustion analysis.

In a continuation of the earlier work, the same water-washed sample, which had been stored in a deep freeze, was further

washed exhaustively with water and was examined by energy dispersive x-ray analysis. In this technique the spectra were acquired from pelletized samples (250 mg under 20 tons for 3 min) with a Kevex Model 7500 operating in the point mode. From the average size (P/B ratio) of the Kα peak for sulfur (at 2.31 kev), measured at several points, a measure of the sulfur content was obtained. By comparison of this value with the corresponding value obtained with the same technique when applied to unwashed steam-exploded wood of known sulfur content (obtained by combustion analysis), a rough estimate of 0.07% was obtained for the exhaustively washed sample. The exhaustive water washing had, therefore, apparently caused little or no further reduction in the sulfur content.

Extraction with acetone-water removed much of the lignin and reduced the sulfur content in the insoluble cellulose-rich residue to approximately 0.04%, as estimated by this same technique. Partial evaporation of the acetone-water resulted in the precipitation of a less soluble lignin-rich fraction of sulfur content 0.07%. Further evaporation to dryness gave a more-soluble lignin-rich fraction of enriched sulfur content 0.17%, suggesting that much of the sulfur in the water-washed steam-exploded wood was associated with the lignin. Although approximately three times as much sulfur was present in the original water-soluble fraction, as was present in the insolubles, no attempt was made to fractionate the solubles and locate the sulfur.

Combination SO_2-H_2O_2 Treatments

As part of Objective 2 for the 3rd year of the contract, the effect of a secondary H_2O_2 treatment after a steam treatment catalysed by SO_2, was examined. The procedure and analyses are summarized in Table 19. The SO_2 treatment (120 s/210°C), in the canister, was comparable to that of Experiment 3 of Table 12, which had given the maximum recovery of xylose in the water solubles, and had solubilized 4.9% of the cellulose or other glucan. One difference was that in this combined SO_2-H_2O_2 experiment commercial chips of aspenwood were used. The water and alkali washes were similar to those of the hydrogen peroxide experiment of Table 4. The hydrogen peroxide treatment was similar to that of Gould which was used in preparing Pulp #1 of Table 4.

Duplicate enzymatic hydrolyses were done, at each of four enzyme loadings, on the insoluble fractions of the SO_2-catalyzed steamed wood remaining after washing washing (WI), alkali washing (WIA) and H_2O_2 treating (WIA-H_2O_2). The mean yields of glucose obtained are listed in Table 20. The four enzyme loadings (15.1, 12.3, 9.4 and 6.0) were all per gram of total substrate, in order to avoid the delay (for analysis) which would have been necessary if equal loadings per gram of cellulose content were to be used. Consequently, when eventually converted to a per-gram-of-cellulose basis (Column 3, Table 20), the enzyme loadings of WI (with its relatively lower cellulose content) can be seen to be significantly higher than those of WIA and WIA-H_2O_2 (with their relatively higher and almost equal cellulose contents).

Table 19. Effect of Water-Washing, Alkali-Washing and Peroxide Treatment on Chemical Composition of Aspenwood Impregnated with 1.6 Percent of SO_2 and Heated 120 min. in Steam at 210°C, with explosion.

		Equivalent dry weight (g)	Composition: Analyses (%) L[1]	H[2]	HPLC[3] G	HPLC[3] X	Original wood basis L[1]	H[2]	HPLC[3] G	HPLC[3] X
	aspenwood	100	19.8	47.1	43.2	21.9	(same as analysis)			
SO_2, steam	↓		3.0							
	steam-heated wood		22.7							
water wash	↓									
	water-washed steamed wood	61.6	28.6	68.3	62.6	0.64	17.6	42.1	38.6	0.39
			1.7				1.0			
			30.3				18.7			
alkali wash	↓									
	alkali-insol. steamed wood	43.3	5.0	92.4	85.9	0.67	2.2	40.0	37.2	0.29
			0.3				0.1			
			5.4				2.3			
H_2O_2 treatment	↓									
	peroxide-treated steamed wood	42.1	1.9	94.6	86.6	0.73	0.8	39.8	36.5	0.31
			0.3				0.1			
			2.2				0.9			

[1] Value obtained on Klason lignin analysis + acid-soluble lignin = total apparent lignin.
[2] Acid-soluble lignin analysis is questionable after peroxide treatment.
[3] Hexosan by anthrone method (adjusted to correct for xylan)
Glucan and xylan by HPLC

Table 20. Comparison, at Four Enzyme[1] Loadings, of Enzymatic Hydrolysis of SO_2-catalyzed[2] Steam-Heated Aspenwood (120/210°C) after Water Washing, Water-and-Alkali Washing, and H_2O_2 Treatment

Substrate	FPU/g of total substrate	FPU/g of cellulose[3]	Glucose yield (% of theoretical) 6h	12h	24h	48h	72h
WI[4]	15.1	22.1	57.9	80.9	93.3	94.9	97.6
	12.3	18.0	53.1	76.0	88.6	94.9	97.8
	9.4	13.8	43.0	61.5	81.8	91.0	95.8
	6.0	8.8	30.0	43.1	57.1	75.6	85.7
WIA[5]	15.1	16.3	46.0	63.1	80.0	93.3	99.4
	12.3	13.3	42.5	58.1	74.7	90.0	98.8
	9.4	10.2	34.1	46.8	62.6	78.6	89.4
	6.0	6.5	21.9	31.0	42.8	55.7	66.8
WIA-H_2O_2[6]	15.1	16.0	50.2	70.8	89.1	98.7	101.6
	12.3	13.0	47.5	66.5	87.7	98.5	101.7
	9.4	9.9	38.5	55.4	78.9	94.9	101.1
	6.0	6.3	27.2	40.4	56.2	75.6	89.1

[1] Mixture of Novozyme & Celluclast
[2] 1.6% SO_2 on equivalent dry weight of wood
[3] Filter paper units per gram of SHA-WI, SHA-WIA or SHA-WIA-H_2O_2
[4] WI-water-washed steam-heated aspenwood, water-insoluble fraction
[5] WIA-water-and-alkali washed, insoluble fraction
[6] WIA-H_2O_2 - water & alkali-washed & H_2O_2 treated

The effects of enzyme loading, on glucose yield after 6, 12, 24, 48 and 72 hours of enzymatic hydrolysis, are shown in Fig. 20, 21 and 22 for the WI, WIA and WIA/H_2O_2 substrates respectively. In order to compare the glucose yields from WI and from WIA-H_2O_2, at the same enzyme loadings (per gram of cellulose) as had actually been used with the WIA, these desired glucose yields were read from Fig. 20 and 22, and were listed in Table 21. For easier comparison, the WIA data of Table 20 are repeated in Table 21.

Fig. 23 shows the resulting calculated courses of hydrolysis. It can be seen that there was little difference between the WI and WIA curves at equal enzyme loadings per gram of cellulose. What little difference there was, indicated a slightly slower hydrolysis of the WIA at first, which was overcome after about 48 h.

At all four enzyme loadings, the WIA-H_2O_2 gave the fastest and most complete hydrolysis. The difference in glucose yield as a result of peroxide treatment, although it was slight at the highest enzyme loading (16.3 FPU/g cellulose), increased markedly as the enzyme loading was decreased. For example, at an enzyme loading of 13.3 FPU/g cellulose, the H_2O_2 treatment increased the glucose yield obtained with the alkali-washed material from 74.7% (of theoretical) to 88.1% after 24h, or from 90.0% to 98.5% after 48h. At the lower loading of 10.2 FPU/g cellulose, the correspondingly greater increases were from 62.6% to 80.0% after 24h, and from 78.6 to 95.2% after 48h.

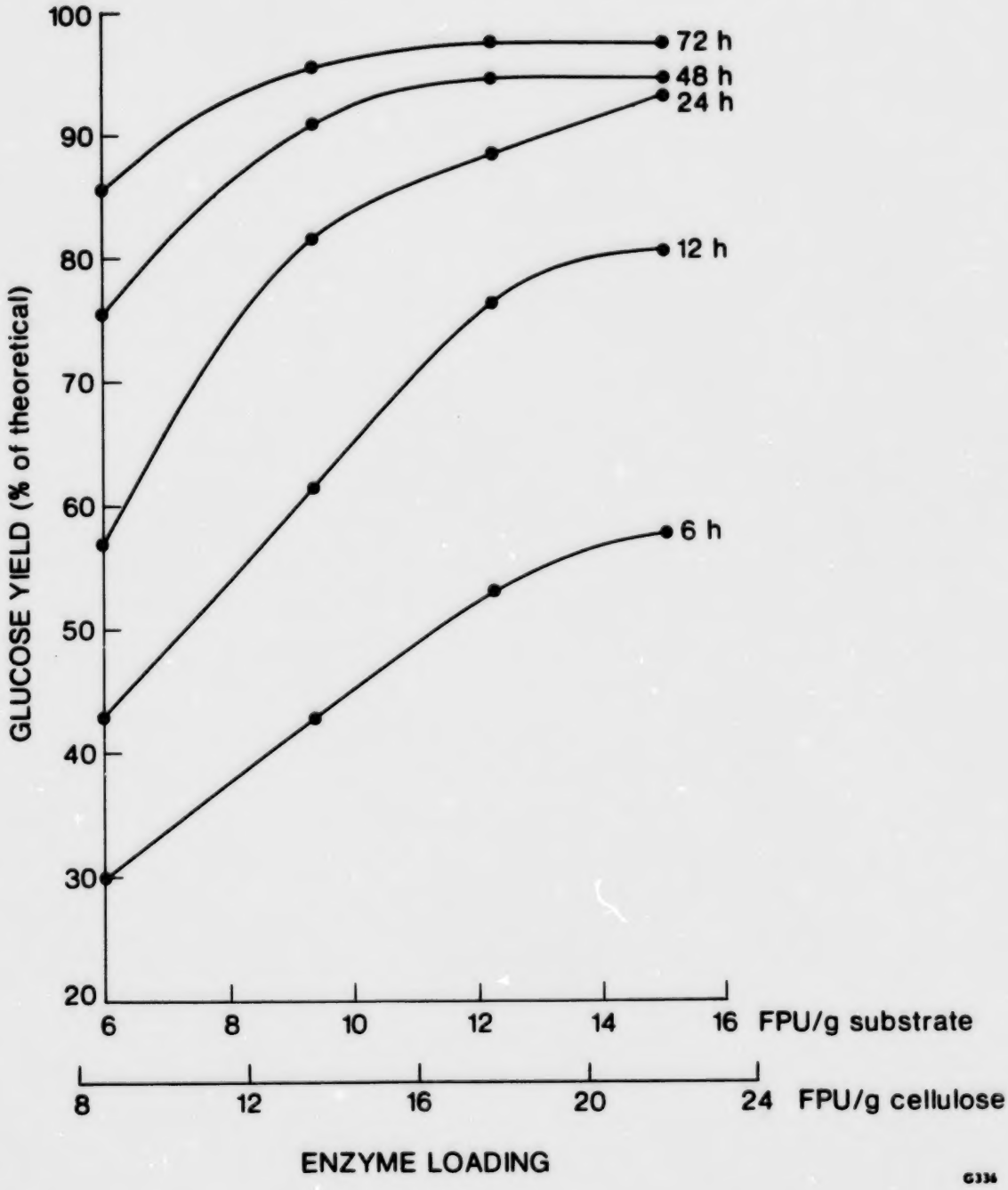


Figure 20. Effect of enzyme loading (E58) on enzymatic hydrolysis of water-insoluble fraction of SO_2-catalyzed steam-heated aspenwood (120s/210°C)

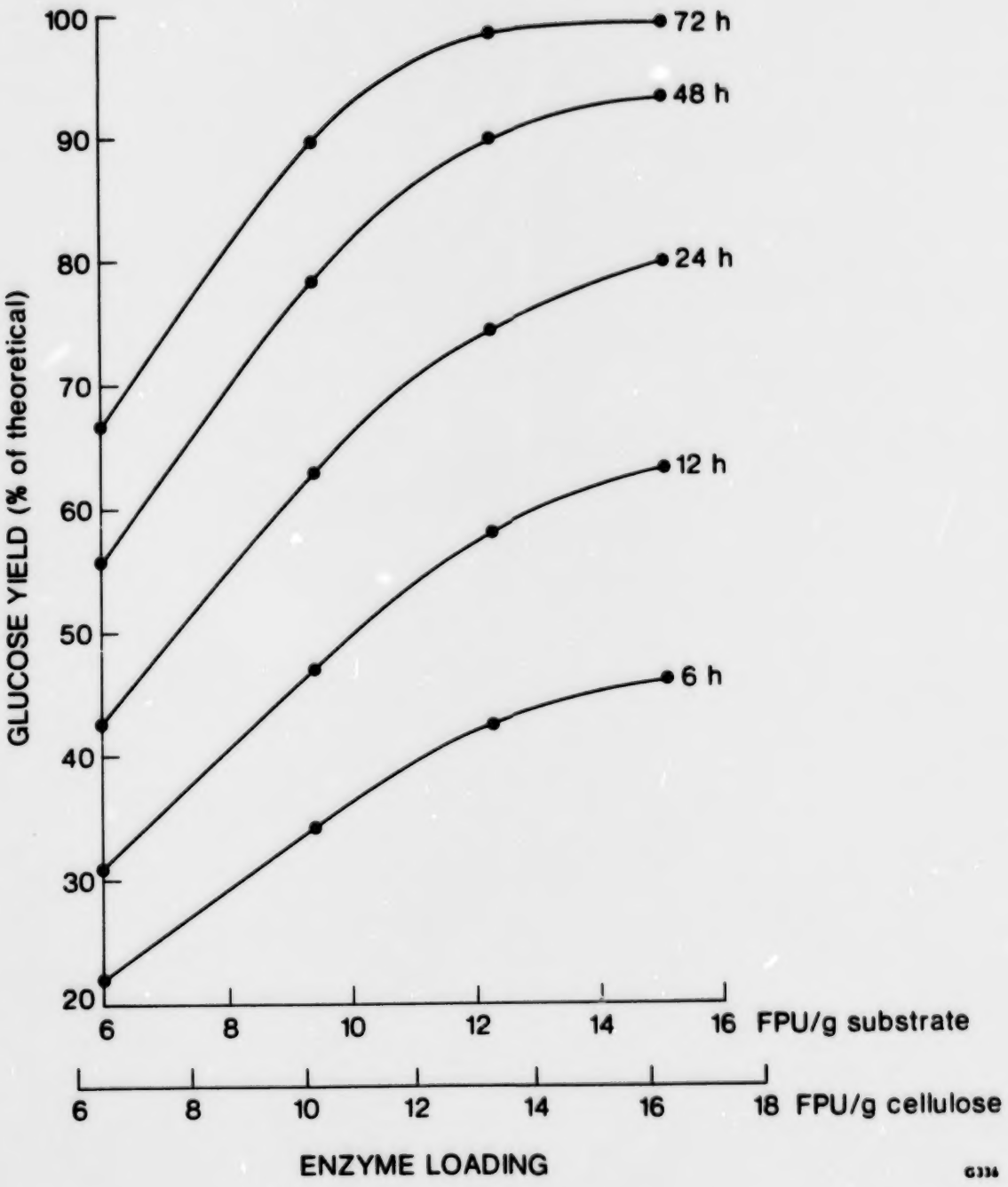


Figure 21. Effect of enzyme loading (E58) on enzymatic hydrolysis of water-and-dilute-alkali-insoluble fraction of SO_2-catalyzed steam-heated aspenwood (120s/210°C)

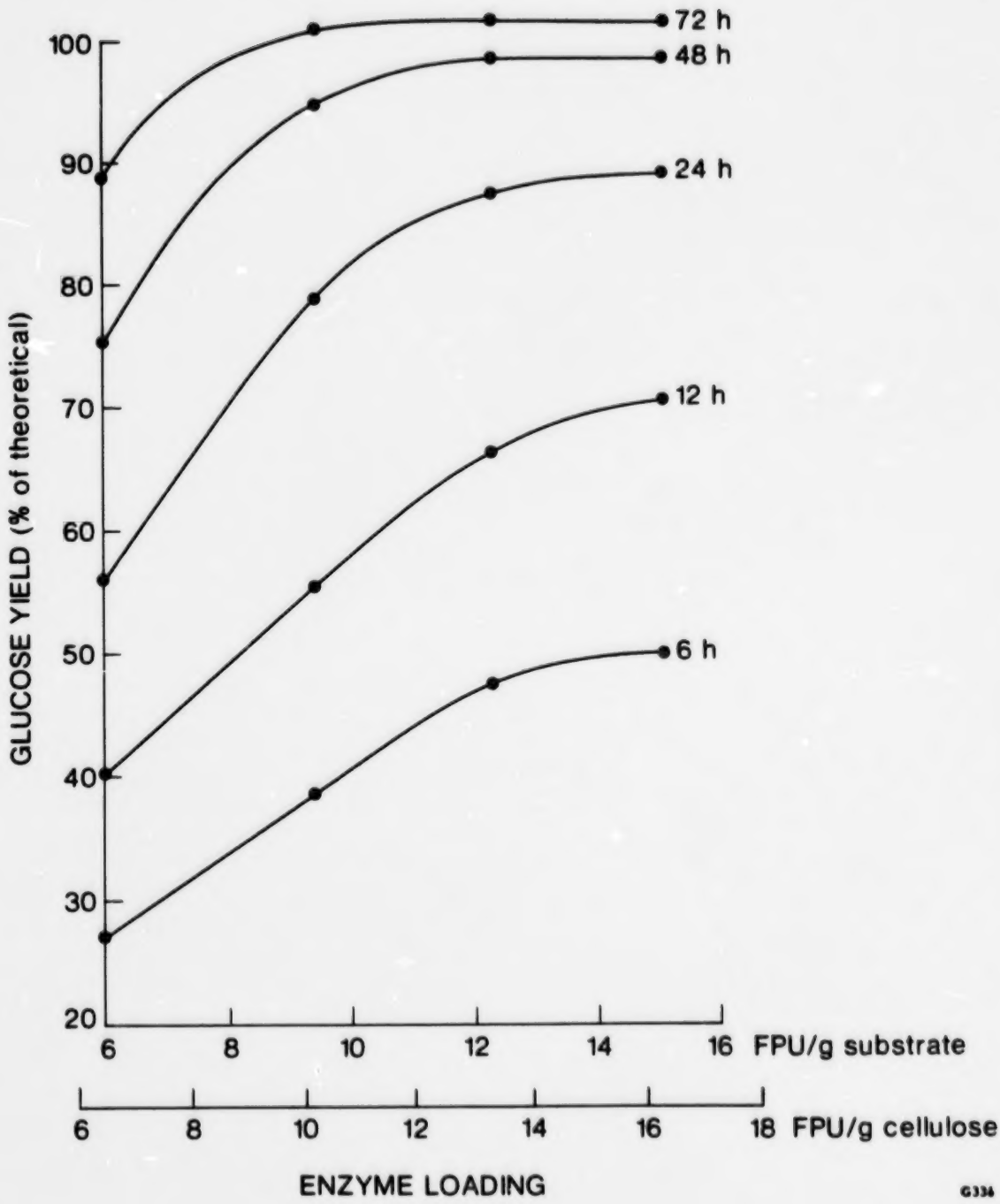


Figure 22. Effect of enzyme loading (E58) on enzymatic hydrolysis of H_2O_2-treated water-and-dilute-alkali insoluble fraction of SO_2-catalyzed steam-heated aspenwood (120s/210°C)

Table 21. Effect of Alkali Washing, and of Alkali Washing Followed by H_2O_2 Treatment of Water-Insoluble Fraction of SO_2-Catalyzed Steam-Heated Aspenwood (120s/210°C) on Enzymatic Hydrolysis at Constant Enzyme Loadings (Expressed per Gram of Cellulose Content) Calculated from Data of Table 20[1]

Substrate	FPU/g of cellulose	Glucose yield (% of theoretical) 6h	12h	24h	48h	72h
WI	16.3	49.5	71.3	86.4	94.0	97.2
	13.3	41.8	59.7	80.2	90.1	95.4
	10.2	33.7	48.0	66.0	81.4	89.7
WIA	16.3	46.0	63.1	80.0	93.3	99.4
	13.3	42.5	58.1	74.7	90.0	98.8
	10.2	34.1	46.8	62.6	78.6	89.4
	6.5	21.9	31.0	42.8	55.7	66.8
WIA-H_2O_2	16.3	50.3	71.0	89.2	98.7	101.6
	13.3	47.9	67.3	88.1	98.5	101.7
	10.2	39.2	56.3	80.0	95.2	101.2
	6.5	27.7	41.1	57.2	77.0	90.4

[1]Calculated by plotting glucose yields from WI and WIA-H_2O_2 (data of Table 20) and reading off the yields at the same enzyme loadings (per gram of cellulose) as those of WIA.

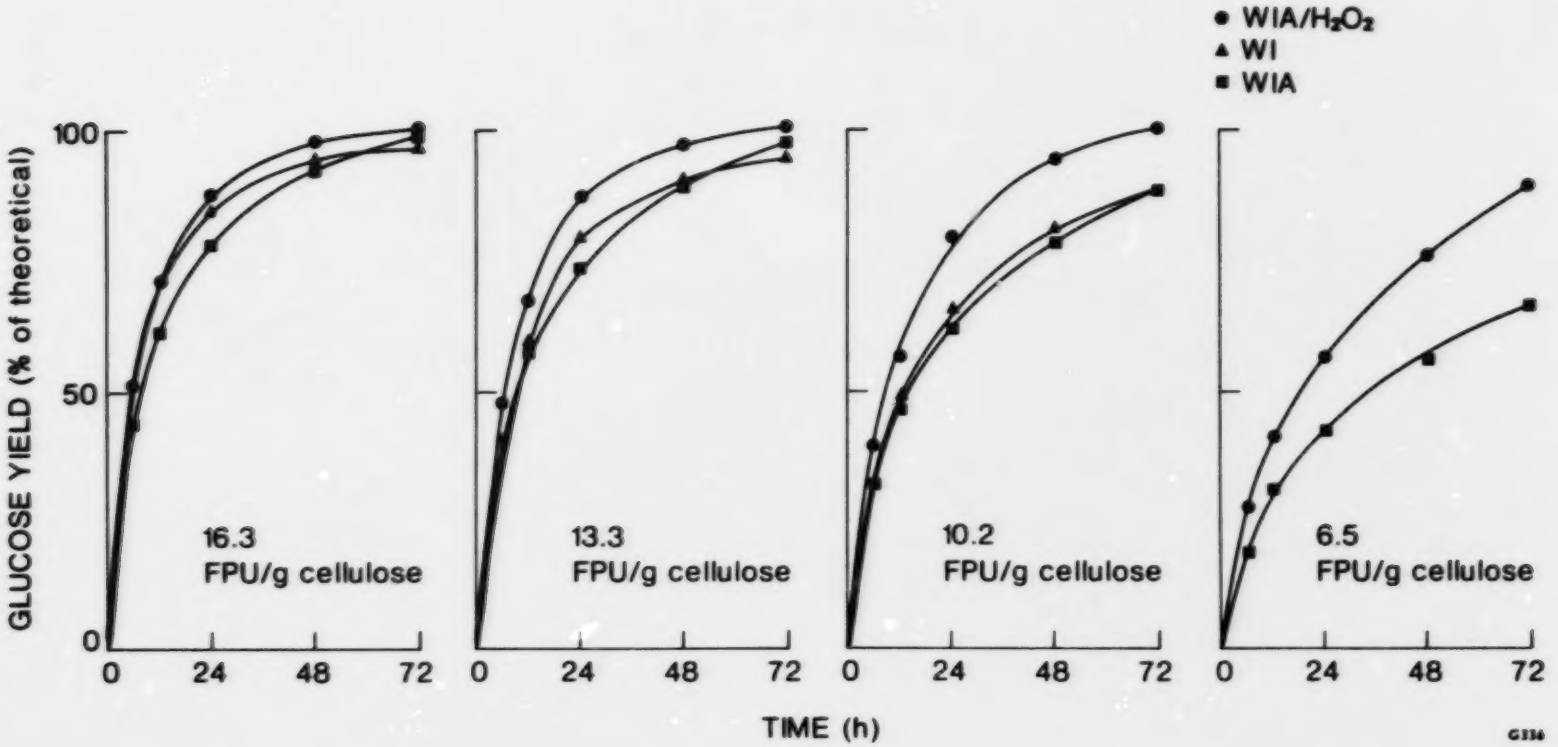


Figure 23. Effect of enzyme loading (Novozyme-Celluclast) on hydrolysis of SO_2-catalyzed steam-heated aspenwood ($1.6SO_2$, 120s/210°C), after water washing (WI), water-and-alkali washing (WIA), and water-and-alkali washing followed by H_2O_2 treatment (WIA/H_2O_2)

In a preliminary experiment, to this SO_2-H_2O_2 experiment described above, duplicate enzymatic hydrolyses of the same three substrates were carried out with the same enzyme mixture (Novozyme-Celluclast) at a single loading of 18 FPU/g of total substrate. In order to check the completeness of the enzymatic hydrolyses, the enzymatic residues after 72h were subjected to Klason lignin hydrolysis. The amount of the resulting glucose in the Klason lignin filtrates was measured by HPLC. The results indicated that the residual cellulose, in the enzymatic residues of WI, WIA and WIA-H_2O_2, had amounted to only 1.9, 3.3 and 0.66%, respectively, of the cellulose originally present in these three substrates. Enzymatic hydrolysis had been essentially complete.

It can be concluded that the combination of treatments (SO_2-catalyzed steaming, followed by peroxide treatment) produced a better substrate for enzymatic hydrolysis, than had the SO_2-catalyzed steaming along. Table 19 shows that this improvement was obtained at the expense of virtually no loss of cellulose, the content of which decreased only marginally from 40.0 to 39.8% (original wood basis) as determined by the anthrone method, or from 37.2 to 36.5% as determined by HPLC. Furthermore, the consumption of peroxide was apparently small, (although it was not measured directly), since the WIA decreased in weight by only 1.2% (original wood basis), largely as a result of a 1.4% decrease in the apparent Klason lignin content.

Effect of Content of Residual Alkali-Insoluble Lignin on Enzymatic Hydrolysis

As shown earlier in Fig. 14 of this report, the rate and extent of enzymatic hydrolysis of the cellulose component of aspenwood increased with increasing steaming times from 20-180s at 240°C. Table 7 showed that substantial increases in saccharification yield had occurred even after the xylan content had been reduced to below 2% of the alkali-washed sample (at 80s). Between 80 and 180s, however, the apparent Klason lignin content continued to decrease, over a much larger range, from 8.4 to 2.1%. It appears, therefore, that the alkali-insoluble fraction of the residual lignin (not the total lignin) plays a more important role than does hemicellulose, in protecting the cellulose from enzymatic attack. The alkali-soluble lignin, after recovery by precipitation with dilute acid, was shown by gel-permeation chromatography to have a molecular weight of only approximately 7000. This relatively low-molecular-weight lignin, which is apparently free or is less-rigidly associated with the carbohydrate, has little or no effect on enzymatic hydrolysis.

When the steam-treated (20-180s at 240°) and alkali-washed aspenwood fractions (SHA-WIA) of Table 7 were subsequently treated with H_2O_2 (at pH 11.5), products (SHA-WIA/H_2O_2), were obtained with the compositions shown in Table 22. The residual apparent Klason lignin and residual xylan contents, on an original wood basis, are listed in Table 23 together with the enzymatic hydrolysis yields and with the corresponding data for the same materials before peroxide treatment. These residual

Table 22. Effect of Time of Steaming at 240°C, without Explosion, on Composition of Water-and-Alkali-Insoluble Fraction after Treatment with H_2O_2

Sample	Steaming time (s)	Recovery yield (% on original wood)	Analysis (% of sample)[1] Apparent Klason lignin	Acid-soluble lignin	By HPLC Glucan[2]	By HPLC Xylan	By anthrone Hexosan
SHA-WIA H_2O_2	20	57.2	11.1	2.1	72.9	11.3	75.7
	40	53.0	9.3	1.6	78.0	6.8	81.9
	80	49.5	4.1	0.8	83.1	1.6	84.7
	120	46.2	1.8	0.5	87.7	1.4	91.0
	180	45.3	0.9	0.3	85.0	0.6	88.8

[1] To convert to original-wood basis, multiply by recovery yield

[2] Glucan calculated from glucose and cellobiose

Table 23. Effect of Steaming Time on Contents of Residual Apparent Klason Lignin and Xylan, Remaining in Water-and-Alkali-Insoluble Fraction of Steamed Aspenwood before and after H_2O_2 Treatment, and on the Rate and Extent of Enzymatic Hydrolysis

Fraction	Steaming time (s)	Composition: Klason lignin (% of sample)	Composition: Klason lignin (% of original wood)	Composition: Xylan (% of sample)	Composition: Xylan (% of original wood)	Enzymatic hydrolysis: Glucose yield (% of theoretical)[1] 8h	24h	48h	72h
SHA-WIA	20	15.5	9.4	9.3	5.7	30.1	44.5	52.8	57.1
	40	12.6	7.0	6.8	3.8	31.2	46.9	56.5	68.0
	80	8.4	4.2	1.7	0.9	37.8	60.0	71.0	85.3
	120	3.5	1.6	1.0	0.5	42.5	70.9	84.7	94.0
	180	2.1	1.0	0.6	0.3	44.0	76.2	91.5	108.0
SHA-WIA/ H_2O_2	20	11.1	6.3	11.3	6.5	37.9	57.0	68.2	80.8
	40	9.3	4.9	6.8	3.6	37.4	58.7	68.8	84.4
	80	4.1	2.0	1.6	0.8	43.9	73.9	84.7	91.6
	120	1.8	0.8	1.4	0.6	50.6	81.0	93.3	103.9
	180	0.9	0.4	0.6	0.3	48.4	79.6	92.4	91.9

[1] Theoretical yield calculated from anthrone hexosan values of Tables 7 and 22 Glucose yield includes both glucose and cellobiose

lignin and xylan contents before and after peroxide treatment are also shown as bar graphs in Fig. 24.

Fig. 24 shows that, after only 20s of steaming, slightly more than half of the Klason lignin of the original wood was removed by subsequent extraction with water and dilute alkali, compared with a proportionately greater two-thirds of the xylan.

The lignin content of the original wood, however, was greater than the xylan content, with the result that lignin and xylan were removed (in almost equal weights) (per 100g of original wood). Fig. 25 shows that lignin and xylan continued to be removed at almost equal rates until after 90s of steaming, when the xylan removal was almost complete.

Fig. 24 shows significant decreases in the lignin content, at each steaming time, as a result of peroxide treatment. In contrast, no corresponding significant decrease in xylan content occurred. It is unlikely that the decreases in Klason lignin content, as a result of peroxide treatment, are in significant error. The samples, as a result of the peroxide treatments, did lose weight (but did not lose xylan) and were enriched in hexosan, as would be expected.

In order to check the possibility that the cellulose accessibility was controlled by the content of residual alkali-insoluble lignin, the glucose yields listed in Table 23 were plotted against the corresponding apparent Klason lignin contents for both SHA-WIA and SHA-WIA/H_2O_2. The resulting points are reasonably well described by the four lines drawn in Fig. 26.

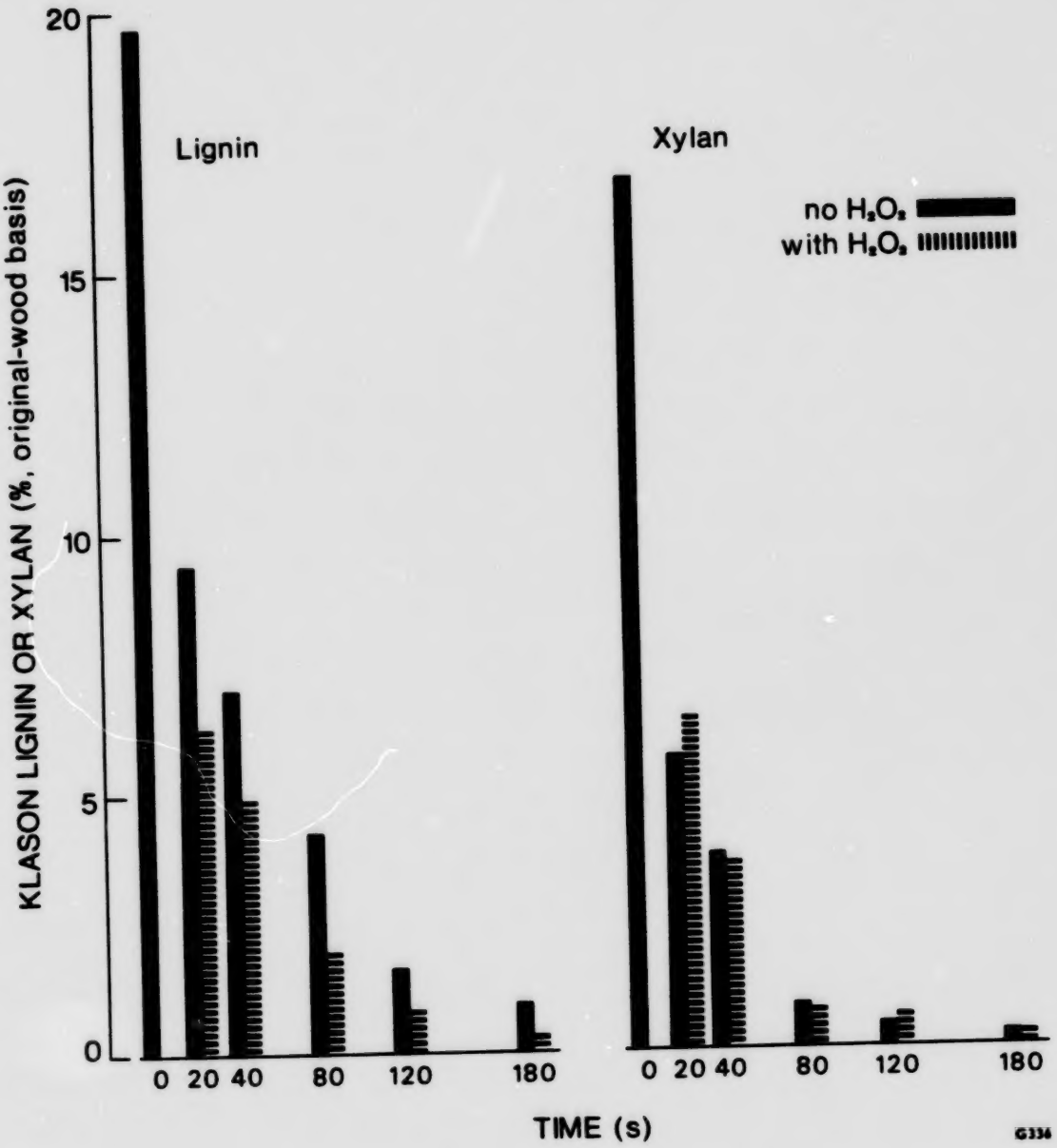


Figure 24. Effect of time of heating of aspenwood in steam at 240°C, on the content of apparent Klason lignin and of xylan remaining in the steamed wood after washing with water and dilute alkali, and after a further treatment with H_2O_2

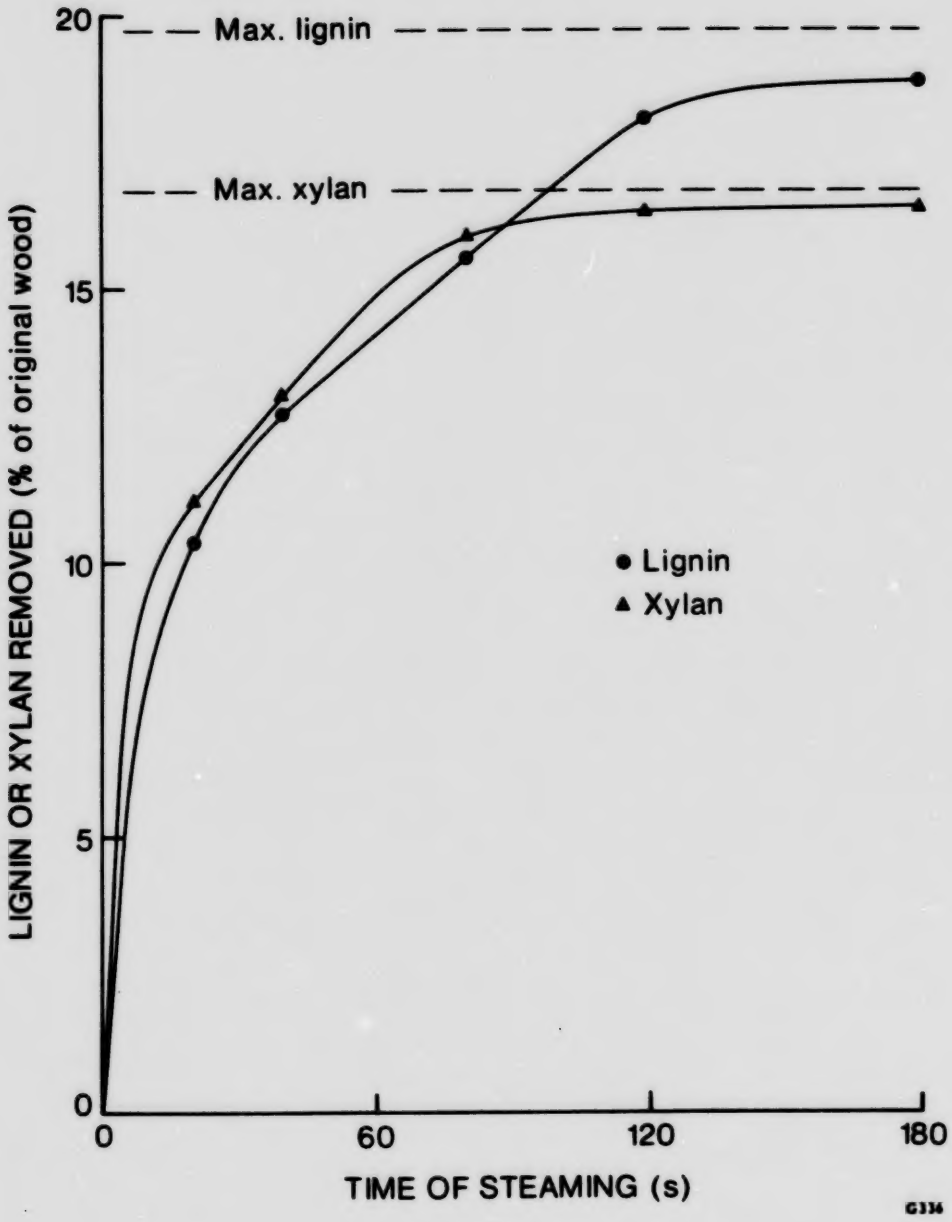


Figure 25. Removal of lignin and xylan from aspenwood during steam treatment at 240°C followed by dilute alkali extraction

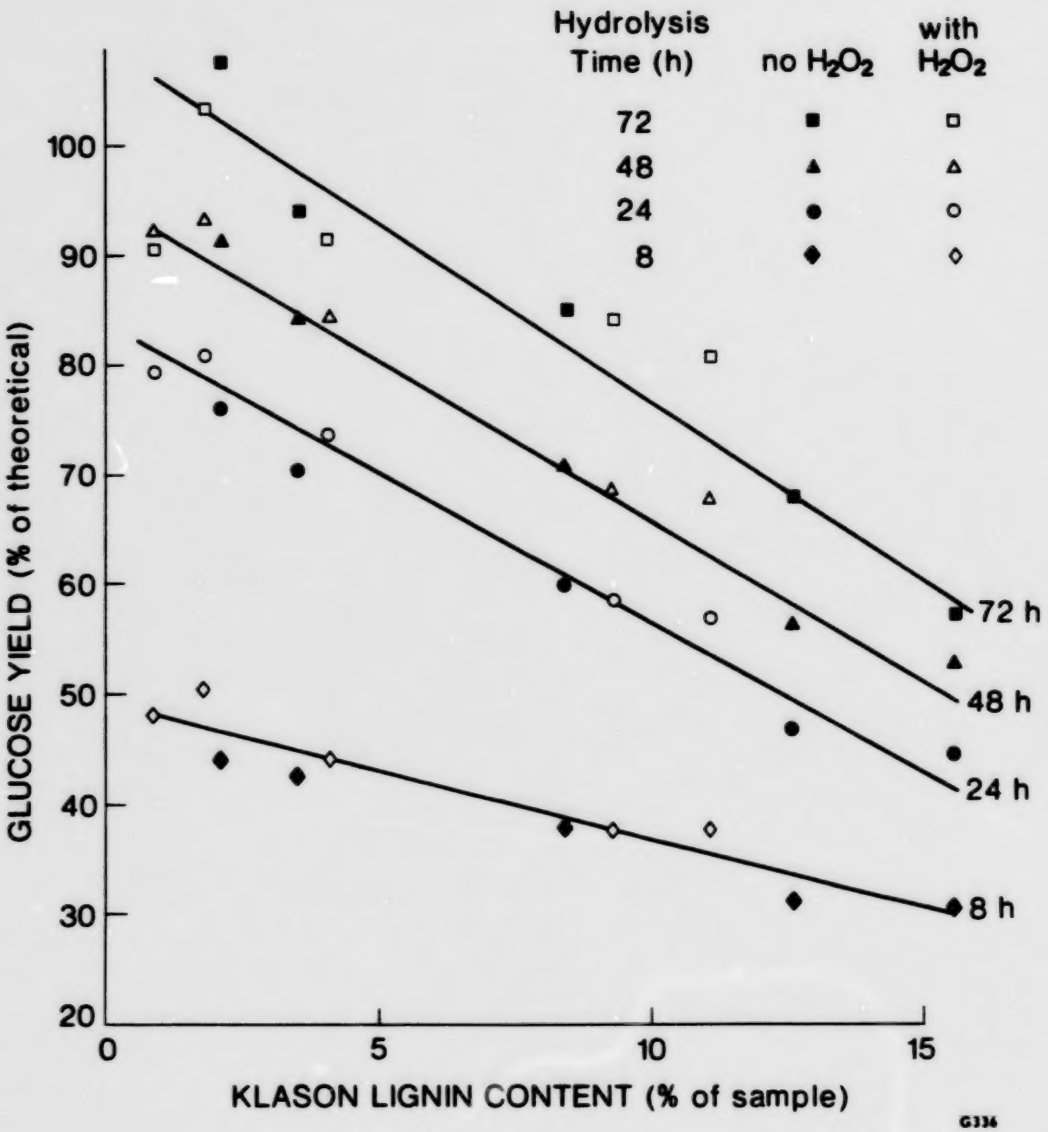


Figure 26. Relationship between apparent Klason lignin content of water-and-alkali-washed steam-heated aspenwood, with or without subsequent treatment with H_2O_2, and the glucose yield obtained on enzymatic hydrolysis

These lines, one for each time of hydrolysis, are approximately straight over the entire range of lignin contents (1-15% of sample weight).

Furthermore, the data for both SHA-WIA and SHA-WIA/H_2O_2 were fitted by the same lines. This means, that it did not matter whether the lower lignin contents were obtained by longer steaming time or by H_2O_2 oxidation. The improvements in hydrolysis yields were the same. It also means that virtually all of the improvement resulting from peroxide treatment is the result of the reduction in content of alkali-insoluble lignin. The apparent tendency, for the peroxide points to be slightly above the best line drawn through the non-peroxide points, may result from the fact that no additional alkali extraction was done after the peroxide treatments. Although these treatments were done at pH 11.5, it is possible that slightly more of the oxidized lignin would have been removed by the more strongly alkaline 0.1N NaOH normally used in the dilute alkali extractions.

During the early stages of this work, before the continuing decrease in the content alkali-insoluble lignin with increasing time of steam treatment had been observed, it was believed that increased accessibility resulted from the removal of xylan. For comparison, therefore, the hydrolysis data of Table 23 were also plotted against the content of residual xylan. Fig. 27 shows that the glucose yield did rise with decreasing xylan content. The points from the samples treated with peroxide, however,

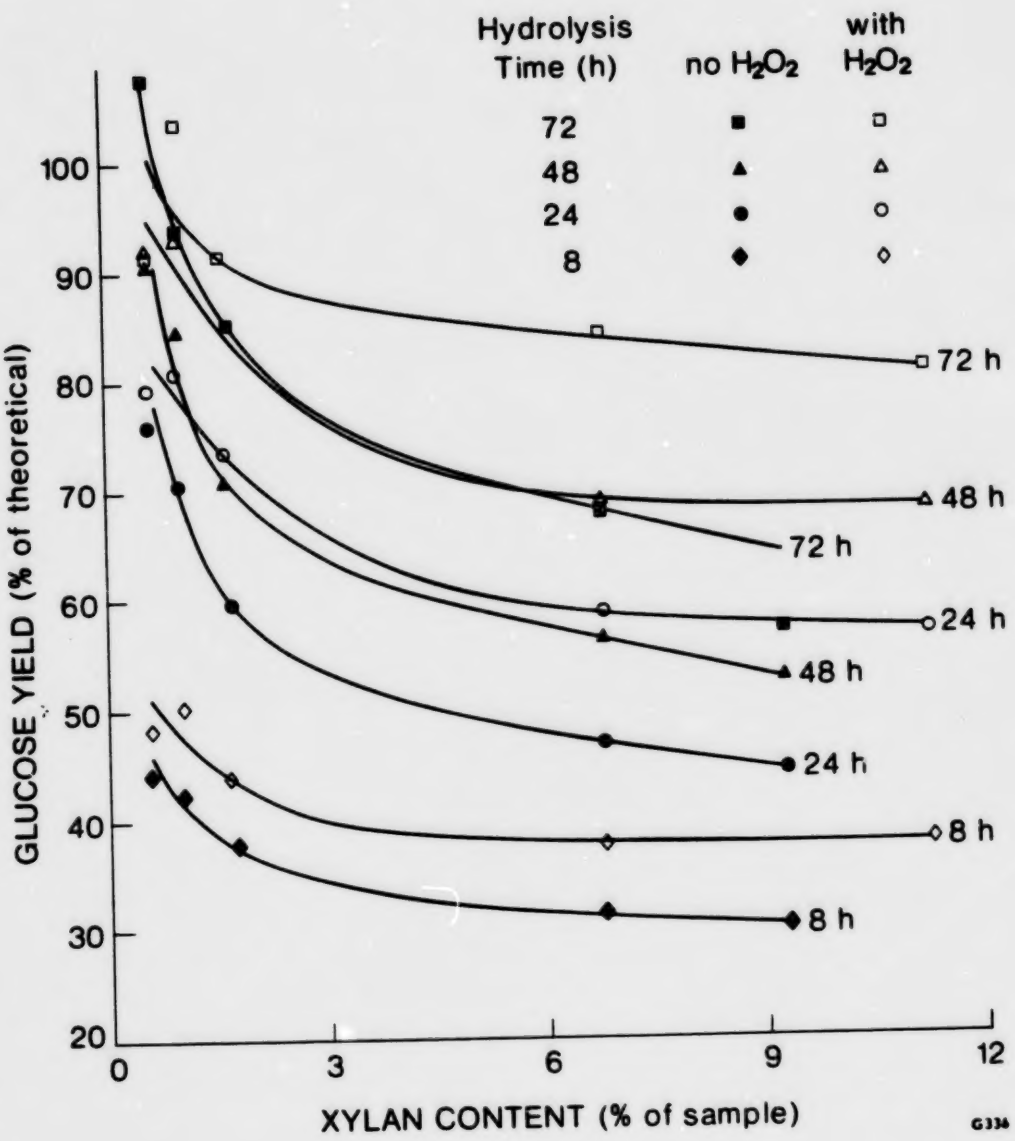


Figure 27. Glucose yield obtained on enzymatic hydrolysis of steam-heated, water-and-alkali-washed aspenwood, both before and after secondary treatment with H_2O_2, plotted against residual xylan content of the sample

clearly do not lie along the same lines as the points from the corresponding samples which had not been treated with peroxide. Peroxide treatment raised the lines because the accessibility was increased by some means other than by xylan removal.

Fig. 27 also shows a pronounced upward curving of the lines as the xylan content was reduced only slightly, from the already low value of of 1.7% to 0.6% of the sample weight. It is concluded that the corresponding decrease in lignin content, which was approximately six times greater than this slight decrease in xylan, was the real cause of the improved hydrolysis yield.

In a further experiment, not described here in detail, approximately 90% of the lignin of aspenwood chips was removed, essentially as in the preparation of chlorite holocellulose (29) (except for the larger particle size). The resulting "low-lignin aspenwood" (LLA) retained virtually all of the original xylan of the wood. The low apparent-Klason-lignin content, of only 2.5% (at least part of which was presumably alkali-soluble), was only slightly higher than the 1.6% remaining in normal aspenwood after long steaming (180s/240°C), and water and alkali washing (SHA-WIA-180s). The chemical compositions, and glucose yields obtained on enzymatic hydrolysis, are listed in Table 24 for both SHA-WIA-180s and LLA, and for the water-insoluble fraction remaining from LLA after steam treatment at 240° for 20s (SHLLA-WI-20s).

Table 24 permits a comparison of the enzymatic hydrolysis of these three materials which had similar very low lignin contents,

Table 24. Comparison of Lignin Removal by Chlorite Treatment, with Lignin Removal by Steam Treatment at 240°C and Alkali Extraction, on Residual Xylan Content and on Enzymatic Hydrolysis

Fraction	Composition (% of sample)		Glucose Yield (% of theoretical)			
	Klason Lignin[1]	Xylan	8h	24h	48h	72h
LLA[2]	2.5	26.4	28.8	51.2	62.3	61.2
SHLLA-WI[3] (20s)	1.6	5.0	43.7	74.1	91.3	98.2
SHA-WIA[4] (180s)	2.1	0.6	44.0	76.2	91.5	108.0

[1] apparent Klason lignin

[2] "low-lignin aspenwood" from chlorite oxidation of chips (resembles incompletely delignified holocellulose)

[3] "steam-heated low lignin aspenwood, water-insoluble fraction"

[4] "steam-heated aspenwood, water-insoluble fraction, alkali washed"

but very different xylan contents. Removal of most of the lignin, during the preparation of LLA, increased the glucose yield on subsequent enzymatic hydrolysis, from what would have been a negligible yield (for untreated aspenwood), to over 60% of theoretical for LLA. This increase, however, was still much smaller than that obtained with SHA-WIA-180s, where, during the preparation, both the lignin and xylan contents were reduced to very low levels (2.1 and 0.6%, respectively), and where essentially quantitative enzymatic hydrolysis was obtained.

Steam treatment of LLA for only 20s, followed by water washing, reduced the residual xylan content to 5.0% (still well above that of SHA-WIA-180s), and resulted in essentially quantitative enzymatic hydrolysis. In fact, the glucose yields obtained after 8, 24, and 48h of hydrolysis were virtually identical with those obtained from SHA-WIA-180s and, if plotted in Fig. 26, would have lain close to the 8, 24 and 48h lines. Furthermore, longer steam treatments of LLA, for 40, 80, 120 and 180s, although they reduced the residual xylan to 3.4, 0.5, <0.5 and <0.5% respectively, did not significantly improve the enzymatic hydrolysis. In fact, the glucose yields decreased slightly for steaming times longer than 40s.

It is concluded that for alkali-insoluble lignin contents below 15% of sample weight, it is primarily the lignin (rather than xylan) which reduces the rate of enzymatic hydrolysis. When, however, xylan contents are high, the xylan also reduces the rate.

Chum et al (30), in a recent study, correlated glucan digestibility of organosolv pulps of aspen and black cottonwood, with the chemical composition of the pulps. They reported that when the xylan content in the pulp was reduced to below 13%, the digestibilities increased significantly. When xylan removal was complete, very high glucan digestibility was observed, "with a small downward trend at very high Klason lignin content". On the other hand, samples in which xylan removal was not complete also exhibited a marked decrease in digestibility as a function of increased lignin content. They concluded that the kinetics of enzymatic hydrolysis of cellulose can be formally treated as two simultaneous pseudo-first-order reactions in which fast and slow hydrolyses of cellulose occur. The higher the residual xylan content, the smaller is the proportion of the fast-hydrolyzable glucans, and the lower is the ultimate glucan digestibility. Also, for samples containing >13% xylan, the higher the residual lignin content, the smaller is the glucan digestibility.

Chum et al appear to recognize the importance of lignin content in controlling digestibility when xylan removal was incomplete. (In all of their pulps, the xylan contents shown were either zero or were greater than 13%). When xylan removal was complete, however, they appear to believe that lignin content had only a small effect on digestibility, in contrast to the results of the present work at very low residual xylan contents.

It is suggested that in organosolv pulping, as in steam treatment, it is not the total residual lignin content that is important, but it is that part of the lignin that can restrict

swelling. It is the content of closely associated lignin of higher molecular weight (which is insoluble in dilute alkali or other good lignin solvents) that matters. In organosolv pulping, the partly hydrolyzed lignin of lowest molecular weight is usually removed in solution in the pulping liquor. This removal, however, was not apparently always complete in the work of Chum et al. They used methanol concentrations as low as 30%, (which are not high enough to give good lignin solvents), apparently deliberately to control the extent of lignin removal. It is suggested that the resulting pulps would contain a significant amount of ineffective lignin (analogous to the alkali-soluble lignin of steam-heated aspenwood). Comparison of the relatively high residual-lignin content of such a pulp, with the lower lignin content of a pulp cooked in a better lignin solvent, leads to the wrong conclusion that it is not lignin which is controlling accessibility.

It was of interest to determine whether or not the improvement in enzymatic hydrolysis of an SO_2-catalyzed steam-treated aspenwood, as a result of peroxide post treatment, could also be explained by the drop in the content of alkali-insoluble lignin. In the SO_2-H_2O_2 experiment described earlier in this report, only one SO_2-catalyzed, water-and-alkali-washed product (apparent Klason lignin content 5.0%, Table 19) had been prepared and had been treated with H_2O_2. The resulting single oxidized product had an apparent Klason lignin content of 1.9%. The corresponding two hydrolysis curves, shown in Fig. 28 for these two substrates, were plotted using the data of Table 20 obtained at the highest

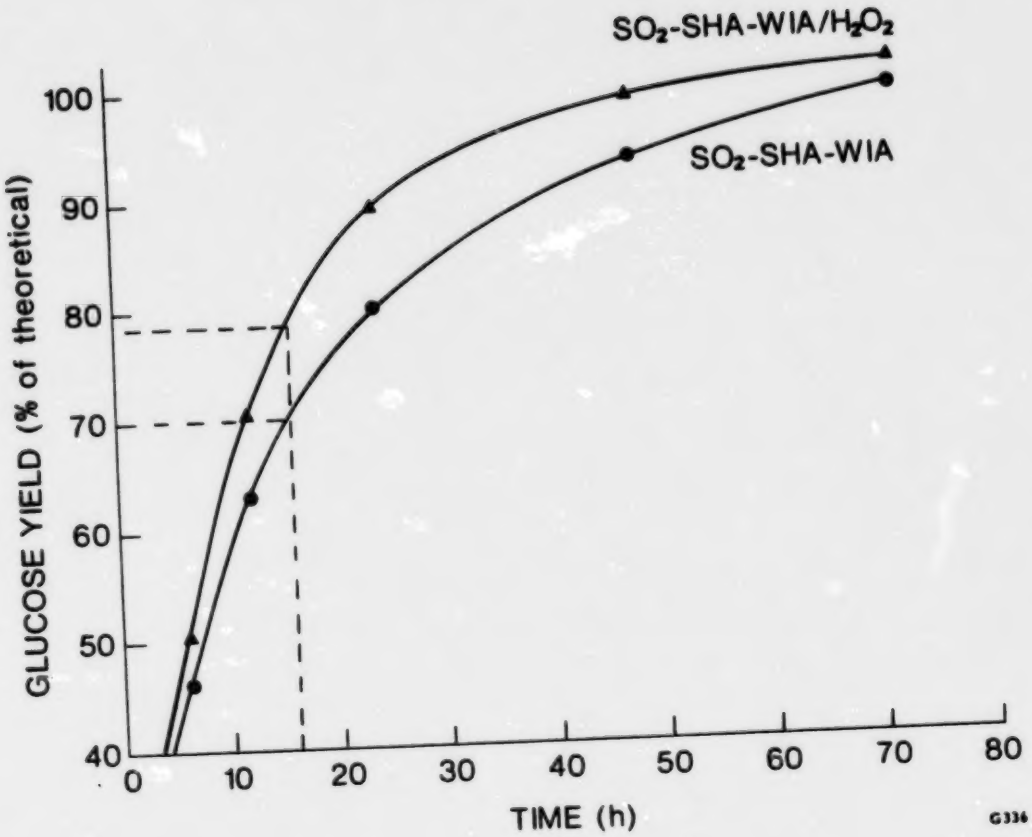


Figure 28. Enzymatic hydrolysis curves for water-and-alkali-washed SO_2-catalyzed steam-heated aspenwood, before and after treatment with H_2O_2 - enzyme loading : 15.1FPU/g total substrate

enzyme loading used (Novozyme-Celluclast, 15.1 FPU/g total substrate).

In order to compare the differences in glucose yield between these two curves, with the differences to be expected solely from the difference in lignin content, the data of Fig. 26 (no SO_2) were used. From each of the 4 lines of Fig. 26 (for 8,24, 48 and 72 h of hydrolysis), the glucose yields to be expected for substrates of the same time lignin contents (5.0 and 1.9%) were read and recorded in Columns 2 and 3 of Table 25.

In order to compare these lignin-related improvements in glucose yield, with the peroxide induced improvements of Fig. 28, and to make the comparisons at the same degrees of completion of the hydrolysis, glucose yields were read from the upper curve (SO_2-SHA-WIA/H_2O_2) of Fig. 28 at the same hydrolysis times which were indicated by the lower curve (SO_2-SHA-WIA) to give the yields listed in Column 2 of Table 25. For example, Column 2 of Table 25 (no-SO_2 case) shows a glucose yield of 70.2% after 24 hours of hydrolysis. The lower curve of Fig. 28 shows that (in the SO_2 case) this yield of 70.2% corresponds to an hydrolysis time of 15.8 h. For this same time of 15.8 h, the upper curve shows a glucose yield of 78.7%, which is listed in Column 6.

Columns 6 and 3 of Table 25 are virtually identical in 3 of the 4 yields. The one discrepancy clearly involves an analytical error (over 100% of theoretical yield). The close agreements indicate that the improvement in enzymatic hydrolysis, as a result of peroxide treatment in the SO_2-H_2O_2 experiment, can be entirely

Table 25. Comparison of the Increase in Glucose Yield from SO_2-catalyzed Steam-heated Aspenwood, as a Result of Peroxide Secondary Treatment, with the Increase Expected as a Result of the Decreased Lignin Content

Data from Fig. 26 (no SO_2)			Data from Fig. 28 (with SO_2)		
Hydrolysis time (h)	Glucose yield (%)[1]		Hydrolysis time[2] (h)	Glucose yield (%)	
	5% Klason lignin	1.9% Klason lignin		5% Klason lignin[3]	1.9% Klason lignin[4]
8	43.0	47.0	5.2	43.0	47.5
24	70.2	78.8	15.8	70.0	78.7
48	80.5	89.7	24.5	80.5	89.7
72	93.1	103.2	47.2	93.1	93.8

[1] Glucose yield (% of theoretical), read from each of the 4 lines of Fig. 27 at the apparent Klason lignin contents of 5.0 and 1.9%.

[2] Hydrolysis time required to give glucose yield listed in Column 5 (of this table), read from lower curve of Fig. 29 (for SO_2-SHA-WIA of apparent Klason lignin content 5.0%).

[3] Glucose yields in this column were chosen to be identical with those in Column 2.

[4] Glucose yields in this column were read from the upper curve of Fig. 29 (SO_2-SHA-WIA/H_2O_2) at the times listed in Column 4, for comparison with the yields in Column 3.

accounted for, by the reduction in lignin content. Although a different enzyme preparation (E58, at 35 FPU/g told substrates) was used in the no-SO_2 case, (compared with Novozyme-Celluclast, at 15.1 FPU/g total substrate in the SO_2 case) both of these enzyme preparations were present in excess.

The conclusion, that delignification was the means by which a secondary peroxide treatment improved the digestibility of SO_2-catalyzed steam-treated aspenwood, implies that the benefit conferred by this additional delignification might be equally-well realized by other methods of delignification. Such methods might include other oxidative treatments, or even simply a more drastic SO_2-catalyzed steam treatment.

Variation of Temperation in SO_2-H_2O_2 Treatments

Aspenwood chips, impregnated with 1.6% SO_2 were subjected to treatments of varying severity by heating in the canister with steam at temperatures from 190 to 230°C, all for the same time of 100s. The product from the treatment at 230° was over-cooked and black, and was not further examined. The recoveries, and results of fractionation and analysis of the other products, are listed in Tables 26 and 27.

Table 26 shows that the largest amount of xylose (in the water-solubles fraction) was obtained from the 100s/190°C treatment, which was the mildest treatment of this experiment. At each successivly higher temperature, the amount of xylose remaining in the water solubles decreased, as a result of degradatin reactions. At the same time, the amount of glucose in the water

Table 26. Effect of Steam Temperature on Recovery and Fractionation of Aspenwood Impregnated with 1.6% SO_2 and Steam Heated[1] for 100s

Steam temperature (oC)	% of original oven-dry wood						
	Recovery (water-solubles + water-insolubles	Water solubles	Water insolubles	Water and alkali insolubles	Composition of water solubles		
					xylose	glucose	acetic acid
190	96.4	33.4	63.0	46.4	15.0	1.8	3.9
200	95.2	32.9	62.3	41.9	14.2	3.8	3.9
210	89.5	32.6	56.9	35.3	11.8	7.4	4.1
220	86.4	36.6	49.9	26.2	7.6	12.9	4.4

[1] Aspenwood chips (90 g equivalent dry weight), impregnated with 1.6% SO_2, steam heated in canister

Composition of original aspenwood: Klason lignin 19.76, acid-soluble lignin 3.31, xylan 16.82, hexosan 47.25

Table 27. Effect of Steam Temperature on the Composition of the Insoluble Fractions, of SO_2-impregnated Aspenwood, Remaining After Washing with Water, with Dilute Alkali, and Subsequently with H_2O_2

Fraction	Temperature °C	Composition (% of fraction)[1]			
		Apparent Klason lignin	Acid-soluble lignin	Cellulose	Xylan
WI	190	27.8	2.0	64.6	2.5
	200	29.7	2.3	63.8	1.2
	210	33.8	2.6	61.1	--
	220	43.9	3.0	49.5	--
WIA	190	4.7	0.4	93.6	--
	200	1.2	0.3	93.8	--
	210	2.4	0.3	93.2	--
	220	3.5	0.2	93.7	--
WIA/H_2O_2	190	1.4	0.4	96.1	--
	200	0.7	0.2	97.6	--
	210	1.4	0.2	95.6	--

[1] To convert to original wood basis, multiply by the % recovery (from ~~Column 4 of~~ Table 26), and divide by 100

Remove "Column 4 of"

solubles increased until, after the most drastic treatment (100s/220°C) it corresponded to about one-quarter of the original cellulose. The HPLC chromatogram also showed increasing amounts of hydroxymethylfurfural in the water solubles.

Table 27 shows that the amount of residual cellulose in the water-insoluble fraction also decreased until, after the most drastic treatment, it amounted to only a little more than half of the cellulose of the original wood. After all the treatments, except the most severe, the apparent lignin in the water-insoluble fraction decreased only slightly (when calculated to an original-wood basis). After the 100s/220°C treatment, it increased very slightly, presumably as a result of pseudolignin formation. Pseudolignin presumably also formed to some extent at the lower temperatures, but was slightly more than balanced by the solubilization of a small part of the original lignin.

In comparing the severity of the 100s/210°C treatment of this series, (as measured by the amount of glucose in the water-solubles fraction), with the severity of the corresponding earlier SO_2-catalyzed treatments described in Table 12, a substantial difference is apparent. In the earlier experiment, at this same temperature of 210°C, the treatment time would have had to be almost 200s in duration (rather than 100s) to produce the same amount of glucose in the water solubles. Both experiments were performed with the same canister and steam gun, and at the same SO_2 level of 1.6% (O.D. wood basis). The only significant difference appears to be in the moisture contents.

In the earlier experiment (Table 12) small chips of MC 51% (moisture-containing basis) were used, whereas larger commercial chips of MC 36% were used in the experiment of Table 26. Higher moisture content, in the absence of added catalysts, was shown in Appendix 1 to an earlier Forintek report (1) to result in longer optimum steaming times.

A comparison of the composition and enzymatic hydrolysis data from the SO_2-H_2O_2 Experiment (Tables 19 and 20), with the composition and hydrolysis data from the Variation of Temperature Experiment (Table 27), provides an opportunity of checking the conclusion at the end of the preceding section of this report. All the samples of Table 27 were excellent substrates. Almost all gave yields above 90% of theoretical within 48h of enzymatic hydrolysis. (None were above 99%, even after 96 h). Only the 6-hour yields are reported here, in Table 98, as a measure of initial hydrolysis rates. Also included in this table are the apparent Klason lignin contents from Tables 19 and 27.

Table 28 shows that the apparent Klason lignin content of 5.0% (repeated from Table 19) was closely matched by the 4.7% obtained in the 100s/190°C treatment (repeated from Table 27). Also, these two WIA materials gave almost equal initial rates of enzymatic hydrolysis). Raising of the steam temperature from 190° to 200° resulted in a drop in the residual apparent Klason lignin from 4.7 to 1.2%, which is comparable to the drop from 5.0 to 1.9% obtained by peroxide treatment. Furthermore, Table 28 also shows that the corresponding similar increases in glucose yield after 6h of enzymatic hydrolysis were from 46.4 to 49.3,

Table 28. Comparison of Additional Delignification in the SO_2-catalyzed Steam Treatment of Aspenwood by Secondary Peroxide Treatment (Table 20), with Additional Delignification by Increased Steam Temperature (Table 27), and Comparison of the Resulting Increases in Initial Rates of Enzymatic Hydrolysis[1]

	WIA[2]		WIA/H2O2[3]	
	Klason lignin[4] (%)	Glucose[5] (% of theoretical)	Klason lignin[4] (%)	Glucose[5] (% of theoretical)
Table 19, (15.1 FPU/g)				
120s/210°C	5.0	46.0[6]	1.9	50.2[6]
Table 27, (18.6 FPU/g)				
100s/190°C	4.7	46.4	1.4	53.4
100s/200°C	1.2	49.3	0.7	49.9
100s/210°C	2.4	44.8	1.4	45.2
100s/220°C	3.5	37.5	--	--

[1] Enzymatic hydrolysis with Novozyme-Celluclast for 6 h

[2] Water-and-dilute-alkali insoluble fraction from SO_2-catalyzed steam treatment in canister (1.6% SO_2)

[3] WIA, after secondary treatment with H_2O_2

[4] Apparent Klason lignin, (% of sample)

[5] Glucose yield after first 6 h of enzymatic hydrolysis

[6] From Table 20

and from 46.0 to 50.2%, respectively. These data support the contention that the improvement in hydrolysis, conferred by peroxide treatment of alkali-washed steam-treated aspenwood (with or without SO_2 catalysis), is the result of further delignification, and that this further delignification and hydrolysis improvement can equally well be accomplished by increasing the severity of the steam treatment.

Table 28 also shows, however, that still further increases in steaming severity (as in the case of 100s/210°C and 100s/220°C) resulted in an increase in apparent Klason lignin in the WIA fraction. This increase appears to have caused the corresponding lower hydrolysis yields (after 6 h) shown in Table 28. It is possible, therefore, to be too drastic. It is noteworthy, however, that the lowest apparent Klason lignin content of 1.2% (from the 100s/200°C treatment) was attained, while still retaining 74% of the original xylan, as monomeric xylose in the water-solubles fraction, and releasing only 7% of the original cellulose or other glucan, as glucose in the water solubles fraction.

The effect of varying the amount of SO_2 used as catalyst, during the steam-heating of aspenwood, is shown in Table 29. In this series of treatments, a constant steam temperature and time (200°C/100s) were used. Table 29 shows that, as the SO_2 loading was increased from 0.5 to 5.0%, both the water insolubles and the water-and-alkali insolubles decreased in amount, at least partly as a result of increased solubilization of the cellulose. It is

Table 29. Effect of Varying the Amount of SO_2, used as Catalyst During Steam-heating of Aspenwood at 200°C for 100 s, on the Subsequent Fractionation and on the Composition of the Water-solubles

Treatment	Product from steam treatment (% of original oven-dry wood)				
			Composition of water solubles		
SO_2 used	Water insolubles	Water-and alkali insolubles	xylose	glucose	acetic acid
0.5	64.1	45.9	12.9	2.8	2.9
1.0	62.3	42.3	15.0	3.9	3.8
1.6	61.1	41.1	14.2	4.8	4.3
2.5	60.7	39.7	14.4	4.3	3.9
5.0	58.5	38.4	13.8	5.2	4.4
1.6[1]	61.2	41.6	14.7	3.8	3.8
1.6[2]	61.3	41.9	15.1	3.1	3.5

[1] Steam treatment was done immediately after SO_2 impregnation, rather than after standing overnight as was the case with all the other treatments

[2] Aspenwood chips largely freed from atmospheric oxygen by flushing repeatedly with nitrogen and standing under nitrogen before SO_2 impregnation; impregnated chips stood overnight in plastic bag stored in nitrogen-filled container; steam gun flushed with nitrogen

surprising, however that the amount of glucose, in the water-solubles, did not increase much (only from 4.8 to 5.2%) as the SO_2 was increased substantially, from 1.6 to 5.0. Also, the amount of xylose in the water-solubles did not decrease much even at 5% SO_2. Raising of the SO_2 concentration does not appear to increase the severity of the treatment as much as did the increasing of the temperature (in Table 26).

Table 29 also shows that the severity of the treatments was reasonably reproducible, similar results being obtained in repeating the treatment at 1.6% SO_2. Also, displacing the air from the chips and from the gun, before SO_2 impregnation and steam-heating, appears to have made little difference.

Carbohydrate Analysis and Enzymatic-Hydrolysis Yields; Reasons for Errors

Erroneously high enzymatic hydrolysis yields (above 100% of theoretical) have been observed and have been discussed earlier in this report. The worst of such errors were obtained when the glucostat method was used in determining the amount of glucose in the enzymatic hydrolysates. When, however, HPLC was used, both in the analysis of the enzymatic hydrolysates, and in the determination of the amount of cellulose or other glucan in the original substrate, enzymatic hydrolysis yields of 5% or more above 100% of theoretical were still often obtained with highly accessible substrates.

In general, there is no problem in the HPLC analysis of enzymatic hydrolysates. When the Novozyme-Celluclast mixture was used,

there was virtually no cellobiose present. A small peak, with a retention time close to that of cellobiose, was, however, present in HPLC chromatograms. It was recognized to be not due to cellobiose, and was not included in the summation of glucose equivalent. It was small in any case, relative to the size of the error in hydrolysis yield.

The erroneously-high (above 100%) enzymatic-hydrolysis yields were found, almost always, to be the result of erroneously-low determinations of cellulose, or other glucan, in the substrate being enzymatically hydrolyzed. The error was sometimes caused by incomplete treatment of the sample in the primary hydrolysis with 72% sulfuric acid, but in this work at Forintek, it has more commonly been the result of incomplete secondary hydrolysis.

It is essential that no crystalline cellulose remains at the end of the primary hydrolysis, because no amount of boiling or autoclaving in the secondary hydrolysis will dissolve it. Failure to decrystallize the cellulose completely, with the 72% sulfuric acid, results in a low cellulose analysis regardless of whether HPLC or the anthrone method is used. In the few cases where enzymatic-hydrolysis yields above 100% of theoretical were obtained, even when the modified anthrone method had been used in the analysis of the substrate, incomplete primary hydrolysis with 72% sulfuric acid was concluded to be the cause. Incomplete hydrolysis during the secondary hydrolysis, however, has been concluded to be the main reason for low cellulose analyses by HPLC. It is also concluded to be the main reason for the

discrepancy between HPLC and anthrone results, since anthrone results are unaffected by incomplete secondary hydrolysis. Incomplete secondary hydrolysis, therefore, leads to excessively high enzymatic-hydrolysis yields, when based on HPLC-determined cellulose contents. [Anthrone-determined cellulose contents can, however, be too high, but only when a large amount of hemicellulose is present, which is almost never the case with water-washed steam-treated hardwoods].

Analysis for cellulose or other glucan, in the water-insoluble fraction of steam-heated wood, has usually been done by HPLC or anthrone methods on the Klason filtrate remaining after Klason lignin analysis. In the HPLC methods, the amount of both glucose and cellobiose were determined, and the equivalent amount of original glucan was calculated, using the hydrolysis-loss factor (HLF) of 1.055 (determined by experiment with a reference sample of glucose). The resulting glucan values, obtained by HPLC, were generally significantly lower than the corresponding values determined by the anthrone method, even when little or no residual xylan or glucomannan was present. For example, in Table 14 the HPLC values ranged from 6.5 to 9.7% lower than the corresponding anthrone values (%, based on anthrone values).

The cellobiose concentrations in Klason filtrates were commonly about one-eighth as large as the corresponding glucose concentrations. It had been assumed that concentrations of cellotriose, cellotetraose and higher oligomers would be much

smaller than that of cellobiose. When, however, a column (Bio-Rad HPX 42A), capable of resolving and quantifying these oligomers, became available, and authentic reference samples were obtained, it was found that the triose and tetraose were sometimes, (but not always), present in amounts as great as were those of the biose. The triose and tetraose were not normally included in the calculation of total cellulose or glucan content, which resulted, therefore, in a low value. The anthrone method, however, always includes any hexose oligomers present.

The effects of variations in the primary and secondary hydrolyses, of the water-insoluble fraction of the SO_2-catalyzed steam-heated aspenwood (preparation and analysis, Table 19), are shown in Tables 30 and 31. Table 30 shows that very little increase, if any, was obtained in the total glucan, as a result of varying the primary hydrolysis. The total glucan content of the sample of SO_2-SHA-WI, as calculated from the actual amounts of cellobiose and glucose determined by HPLC, appeared to increase only from 58.0 to 59.3% as a result of more thorough stirring during primary hydrolysis. Increasing the time of primary hydrolysis, from 2 to 3 hours, appeared to increase the glucan even less, from 58.0 to 58.7%. Increasing the time of secondary hydrolysis, however, from 4.0 to 5.5 hours, increased the apparent glucan content very substantially from 58.0 to 62.3% of theoretical.

Table 31 shows that, in a further experiment, when thorough mixing was used during the primary hydrolysis in each case, the apparent glucan contents determined after 4, 5 and 6 hours of

Remove 2

Table 30. Effect of Prolonging Time of Primary and Secondary Hydrolysis[1] and of Additional Stirring[2] During Primary Hydrolysis, on Amount of Cellobiose and Glucose in Klason Filtrate of SO_2-SHA-WI[3]

Hydrolysis[1]		Sugar produced[2] per g of WI[3]			Total glucan (% of WI)	
primary (h)	secondary (h)	cellobiose (g)	glucose (g)	total equivalent glucose[4] (g)	no HLF[5]	HLF 1.055[6]
2	4	0.0635	0.5775	0.6445	58.0	61.2
		0.0652	0.5760			
2	4	0.0595	0.5936	0.6593	59.3	62.6
extra stirring[7]		0.0627	0.5963			
3	4	0.0624	0.5882	0.6522	58.7	61.9
		0.0608	0.5866			
2	5.5	0.0264	0.6616	0.6926	62.3	65.8
		0.0271	0.6672			

[1] Primary hydrolysis 18-20°C in 72% sulfuric acid. Secondary hydrolysis, refluxing in 3% sulfuric acid at atmospheric pressure

[2] Weights of cellobiose and of glucose actually determined by HPLC, normalized to an initial oven-dry sample weight of 1.0000 g. Actual sample weights ranged from 0.91 to 0.93 g (equivalent oven-dry weights).

[3] WI - water insoluble fraction from SO_2-catalyzed (1.6% SO_2) steam-heated aspenwood (120s/210°C - Table 19.

[4] (Weight of cellobiose x 1.0526) + weight of glucose

[5] Total glucan content calculated without any hydrolysis loss factor (HLF)

[6] Hydrolysis loss factor of 1.055 assumed

[7] Sample of WI stirred more thoroughly with the 72% sulfuric acid during primary hydrolysis

Table 31. Effect of Time of Secondary Hydrolysis, after a 2-hour Primary Hydrolysis[1] with thorough Stirring[2], on Amount of Cellobiose and Glucose in Klason Filtrate of SO_2-SHA-WI[3]

Secondary hydrolysis time[1] (h)	Sample No.	Sugar produced[4] per g of WI[3] cellobiose (g)	glucose (g)	total equivalent glucose[5] (g)	Total glucan (% of WI) no HLF[6]	with HLF[7]	(assumed HLF)
4	I	0.0511	0.6119	0.6657			
		0.0495	0.6193	0.6715			
	mean	0.0503	0.6156	0.6686	60.2		
	II	0.0572	0.6086	0.6688			
		0.0566	0.6188	0.6785			
	mean	0.0569	0.6137	0.6736	60.6		
	Mean of 4	0.0536	0.6147	0.6711	mean 60.4	63.7	(1.055)
	SD of 4	0.0039	0.0053	0.0054			
5	III	0.0279	0.6782	0.7076			
		0.0282	0.6764	0.7062			
	mean	0.0281	0.6773	0.7069	63.6		
	IV	0.0290	0.6799	0.7105			
		0.0306	0.6694	0.7017			
	mean	9.0298	0.6747	0.7061	63.5		
	Mean of 4	0.0290	0.6760	0.7065	mean 63.6	67.1	(1.055)
	SD of 4	0.0012	0.0046	0.0037			
6	V	0.0228[8]	0.4556[8]				
		0.0170	0.7057	0.7236	65.1		
	VI	0.0150	0.7059	0.7217			
		0.0162	0.7161	0.7332			
	mean	0.0156	0.7110	0.7274	65.5		
	Mean of 3	0.0161	0.7093	0.7262	mean 65.3	68.9	(1.055)
	SD of 3	0.0010	0.0060	0.0062		66.9	(1.025)

[1] Primary hydrolysis, 2h at 18-20°C in 72% sulfuric acid
Secondary hydrolysis, reflexing in 3% sulfuric acid at atmospheric pressure

[2] Extra stirring as in Footnote 7 of Table 30

[3] Water insoluble fraction of SO_2-catalyzed (1.6% So_2) steam-heated aspenwood (120s/210°C) - Table 19

Table 31 (continued)

[4] Weights of cellobiose and glucose determined by HPLC, and normalized to an initial oven-dry sample weight of 1.0000 g. Actual sample weights (O.D.) ranged from 0.94 to 0.99 g.

[5] (Weight of cellobiose x 1.0526) + weight of glucose

[6] Total glucan content calculated, without any hydrolysis loss factor

[7] Total glucan content calculated with the assumption of the hydrolysis loss factor in brackets

[8] Value not used in average

Table 32. Recovery of Glucose (as Determined by HPLC) in Klason Filtrate After 2-hour Primary Hydrolysis, and 6-hour Secondary Hydrolysis[1] of Pure Cellobiose and Pure Glucose

Sample	Sample weight (g)	Weight of glucose in Klason filtrate		Calculated HLF[3]
		by HPLC (g)	% of theoretical[2]	
cellobiose	0.7093	0.723	96.84	
	0.6353	0.656	98.09	
			mean 97.47	1.026
glucose	0.9928	0.969	97.60	1.025
	0.9604	--	--	

[1] Primary hydrolysis, 2 hours at 18-20°C in 72% sulfuric acid
Secondary hydrolysis, 6 hours reflex in 3% sulfuric acid at atmospheric pressure

[2] $\frac{\text{Weight of glucose found by HPLC} \times 100}{\text{weight of cellobiose sample} \times 1.0526}$ in case of cellobiose

or $\frac{\text{Weight of glucose found by HPLC} \times 100}{\text{weight of glucose sample}}$ in case of glucose

[3] $\text{HLF} = \frac{100}{\% \text{ of theoretical}}$

secondary hydrolysis were 60.4, 63.6 and 65.3% respectively. Table 31 also shows that the weight of cellobiose, present in the secondary hydrolysate (per gram of original WI) after 4, 5 and 6 hours, decreased from 0.054 to 0.029 and to 0.016 g, respectively, while the weight of glucose increased much more. Clearly, hydrolysis was incomplete after only 4 hours.

In order to determine appropriate hydrolysis-loss factors (HLF) for use after a 6-hour secondary hydrolysis, pure samples of cellobiose and of glucose were subjected to a 2-hour primary and a 6-hour secondary hydrolysis (3% sulfuric acid, reflexing under atmospheric pressure). Table 32 shows that 97.5% of the theoretical weight of glucose was present in the secondary hydrolysate of the cellobiose, as determined by HPLC without the use of an HLF. No residual cellobiose was detected. A similar weight of glucose (97.6% of the original pure glucose sample) was similarly determined in the secondary hydrolysate from the glucose. These values indicate hydrolysis loss factors of only 1.026 and 1.025, for the 6-hour boil, compared with 1.055 determined for glucose after a 4-hour boil.

Near the end of this work, Theander and Westerlund (31) showed that, during the primary hydrolysis, sulfate esters are formed with the carbohydrate. It is important that the secondary hydrolysis with dilute sulfuric acid be sufficient to hydrolyze these sulfate ester groups. They showed that although a 4-hour boil (with 0.4M sulfuric acid) is sufficient to remove all of the sulfate ester groups from xylose, it is not enough to remove all

these ester groups from glucose. They also suggested a 6-hour reflex or 1 hour in an autoclave at 125°C. It is noteworthy, that removal of sulfate ester groups is not necessary when the anthrone method is used.

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APPENDIX 1

Hexosan by Modified Anthrone Method

Solutions

72% H_2SO_4 (24.1 N): This solution, for primary hydrolysis, was prepared by adding 665 mL of concentrated sulfuric acid to approximately 300 mL of distilled water and, after cooling to room temperature, diluting to 1 L in a volumetric flask. The resulting solution was standardized against standard sodium hydroxide, using methyl orange. The concentration was then adjusted to 72 - 0.1% by adding either water or concentrated sulfuric acid, as required.

34.8 N H_2SO_4: This solution, for use as a solvent for the anthrone, was prepared by adding 2000 mL of concentrated sulfuric acid to 120 mL of distilled water in a 4 L beaker. This slight dilution was made, to release some of the heat of hydration and thus to control the extent of the subsequent temperature rise occurring after mixture with the aqueous sugar solutions.

0.362 N H_2SO_4 (1.77%): This dilute sulfuric acid solution, for use in preparing the anthrone blank and the standard glucose solutions, and also for use in diluting aliquots of the Klason filtrates, was prepared by adding 15 mL of 72% sulfuric acid to about 200 mL of water in a beaker and, after cooling to room temperature, diluting to 1 L in a volumetric flask. The resulting dilute sulfuric acid therefore had the same acid concentration as that of the Klason filtrates after they were also diluted to 1 L as described below.

Anthrone Reagent (2 g/L): Anthrone (0.500 g) was weighed into a vial, from which it was dumped into a 250 mL volumetric flask. Residual traces were then rinsed into the flask from the vial with 34.8 N sulfuric acid and, after standing with occasional swirling until almost all of the sparingly soluble crystalline anthrone had dissolved, the flask was filled to the mark with 34.8 N sulfuric acid. The flask was then allowed to stand in the dark for 4 hours, during which time it was inverted a few times to dissolve virtually all of the remaining crystals. The solution was then filtered through sintered glass before use. This anthrone solution was prepared fresh daily.

Anthrone Blank: This solution, for use in the reference beam, was prepared by adding 10 mL of the anthrone reagent to 5 mL of 0.362 N sulfuric acid in a reaction tube. This blank solution was mixed and heated in the boiling water bath for 4 minutes, as described below for the sugar solutions.

Glucose Standard (0.1 mg/mL): Approximately 1.0 g of dry certified reagent grade glucose, weighed accurately on an analytical balance, was dissolved in 0.362 N sulfuric acid and was diluted with more of this dilute acid to 1 L in a volumetric flask. An aliquot of this solution was then diluted 10-fold by pipetting 10 mL and diluting (again with the dilute acid) to 100 mL in a volumetric flask, to give an accurately known glucose concentration of close to 0.1 mg/mL.

Acid Hydrolysis Procedure

This analysis was performed on the diluted filtrates remaining after Klason lignin analysis. For this purpose, the Klason lignin analysis (Tappi Standard T222 os-74) was slightly modified to give, for convenience, a final volume of filtrate-plus-washings of slightly less than one litre.

In the unmodified Tappi standard method, approximately one gram of wood or pulp is subjected to a primary hydrolysis for 2 hours at 18-20^{o}C with 15 mL of 72% sulfuric acid. The resulting mixture is then diluted to approximately 3% in sulfuric acid by the addition of 560 mL of water. This diluted mixture is subjected to a secondary hydrolysis by boiling for 4 hours, after which the acid-insoluble lignin (Klason lignin) is filtered off and washed in the filter with 500 mL of boiling water. The combined filtrate and washings, containing the hydrolyzed carbohydrate, therefore normally has a volume of approximately 1,075 mL.

In the modified procedure employed in this work, which also is suitable as a Klason lignin analysis, the secondary hydrolysis was done in open flasks (i.e. not under reflux), with water being added at intervals as necessary to maintain the volume. Near the end of the four-hour boil, the volume was allowed to drop slightly, so that, after filtering and washing, the total volume was slightly below 1 litre. After transfer to a volumetric flask, the volume was brought up to 1 L by adding water. By this means, the dilution was kept small, and the sugar concentrations in the 1L of this slightly diluted Klason filtrate were still

high enough for HPLC analysis with the Bio-Rad HPX 87H column. An additional dilution, however, was desirable before analysis by the anthrone method.

Dilution of Klason Filtrate for Anthrone Method

An aliquot of the 1 L of Klason filtrate was further diluted in accordance with the following:

A calibration curve of absorbance at 625 nm, against weight of pure glucose in mg, in the absence of any pentose, had a slope of close to 3.33 and was found to be linear at least up to a total weight of 0.5 mg of glucose in the 5 mL of sugar solution used (i.e. glucose concentration 0.1 mg/mL) at which point the absorbance was 1.67. In the presence of xylose or other pentose, however, the pentose interfered by contributing to the total measured absorbance, in an amount which not only increased with increasing pentose concentration (as would be expected), but which also increased with increasing glucose concentration (which was surprising). The enhancing effect of glucose, on the absorbance contributed by xylose, is shown quantitatively in Fig. A-1 and in the equations presented below.

In the presence of pentose, therefore, in order to minimize this interference and to reduce or to eliminate the necessity for a correction, the Klason filtrate was further diluted with 0.362 N sulfuric acid, to a glucose concentration of about 0.03 mg/mL. At this concentration therefore, the 5 mL aliquot, used with 10 mL of the anthrone reagent, contained a total of 0.15 mg of glucose. This weight of glucose gave an absorbance of close to 0.5.

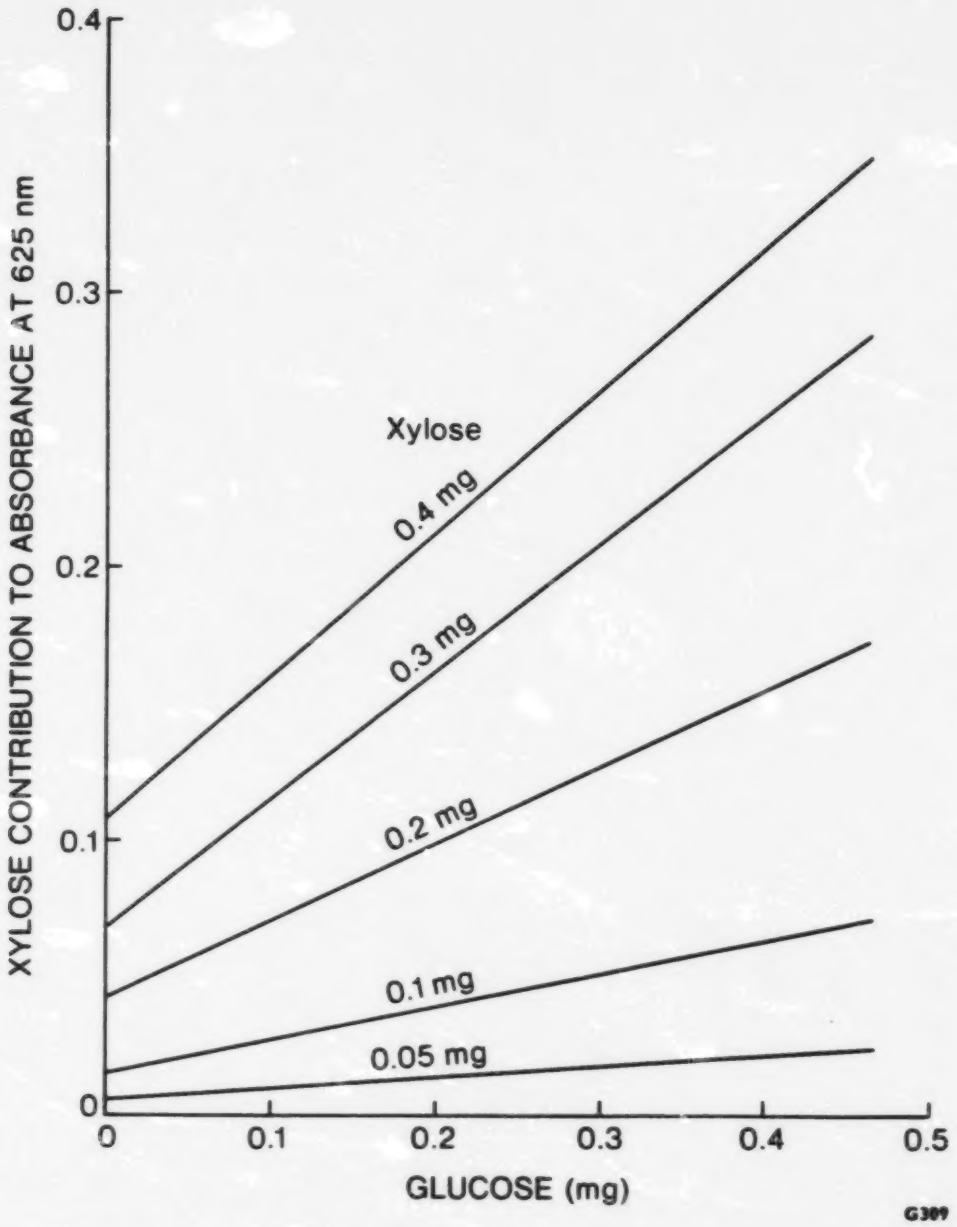

Figure A1-1. Effect of presence of glucose on contribution of xylose to absorbance of mistures of glucose and xylose

One gram of aspenwood, for example, subjected to the Klason hydrolysis procedure, modified as described above, would give 1 L of filtrate and washings containing approximately 0.5 g of glucose and approximately one third of this weight of xylose. Dilution of a 6 mL aliquot to 100 mL would reduce the concentrations of glucose and xylose to 0.03 and 0.01 mg/mL respectively. The 5 mL aliquot of this diluted solution to be used in the anthrone analysis would therefore contain 0.15 mg of glucose and 0.05 mg of xylose.

Under these conditions, the 0.05 mg of xylose would add only 0.011 to the absorbance of 0.5 obtained from the 0.15 mg of glucose. Failure to make any correction for the xylose contribution would, therefore, make the glucose analysis high by only 2.2% of itself, even in the presence of this relatively large proportion of xylose. On the other hand, if this same Klason filtrate were diluted only 5 fold (instead of from 6 mL to 100 mL), the xylose contribution to the absorbance would be relatively much greater (because of the higher glucose concentration) and would be 8.7% as great as the absorbance from the glucose alone. Clearly, however, if Klason filtrates are adequately diluted (to give glucose concentrations of approximately 0.03 mg/mL), and if the samples being analyzed have less than half of the xylose content of the original hardwood (as is the case with water-washed steam treated hardwoods), no correction is necessary because the xylose would contribute less than than 1% to the measured absorbance.

Reaction with Anthrone Reagent

Samples to be analyzed were subjected to Klason lignin analysis as described above. The filtrates from each sample were diluted, also as described above, to reduce the glucose concentrations to approximately 0.03 mg/mL. Aliquots of 5 mL were pipetted, in triplicate into 25 x 150 mm test tubes. (It is important to note that tubes of smaller diameter were found to be unsatisfactory as a result of inefficient mixing of the heavy anthrone reagent with the much lighter aqueous Klason filtrates, even when violently swirled on a vortex stirrer). To each tube was then added (within 10-15s) 10.0 mL of the anthrone reagent from a burette with the tip cut off (to permit faster addition). Immediately after the addition of the anthrone reagent to a tube, the contents were mixed on a vortex stirrer for 5s, and the tube, loosely covered with an inverted 20 mL beaker, was placed in a wire test-tube rack in a vigorously boiling water bath. Addition of anthrone reagent, vortex mixing and insertion into the water bath, were completed in 20s during which time the temperature rose to 100°C or slightly above. A new tube, containing the next sample, was added to the boiling bath every 2 minutes. Each tube was removed after exactly 4 minutes in the bath, and was transferred to a larger test tube rack in an ice-water bath for rapid cooling. After all of the tubes had been in the ice-water bath for at least 15 min., the ice water was replaced with tap water at approximately 25°C, to bring the solutions to room temperature before measuring the absorbances. Because of this schedule, no more than 3 tubes were present in the boiling bath at any one time, and no more than one tube was

added at the same time. The temperature of the bath was therefore easily maintained constant, with the bath at a vigorous boil.

In order to maintain this schedule and because of the viscosity of the anthrone reagent, during the rapid addition of the anthrone reagent from the burette the meniscus was allowed to drop to about 0.15 mL beyond the desired 10.0 mL mark. Experience showed that on standing, the "run-down" from the burette walls brought the meniscus level back to the desired 10.0 mL, showing that the desired volume had been added.

Other methods of anthrone addition were unsatisfactory. Drainage from glass pipettes was too slow. Plastic pipettes (which are designed to contain aqueous solutions) were unsatisfactory because of the much greater density of the anthrone solution in sulfuric acid, which caused dribbling from the orifice. Deliberate layering of the dense anthrone reagent below the aqueous Klason filtrate, followed by rapid mixing to release the heat almost instantly, was also unsatisfactory because of unavoidable and variable mixing at the interface during layering.

During the 4-minute heating, it was important that the test tube rack be of an open construction, (i.e. not to be made from a solid sheet of metal which was found to interfere with the necessary circulation of the bath water and to cause the water in the top of the bath to be several degrees below 100°C). It was also important to prevent air currents in the room from cooling the upper parts of the tubes in the water bath. A simple and

satisfactory arrangement consisted of a tall stainless steel beaker of diameter 20 cm heated by a 2000 w hot plate of diameter 22 cm, with the wire test tube rack positioned so that the tops of the tubes were 4 cm below the top of the walls of the steel beaker.

Glucose Calibration Curve

Among the samples of Klason filtrates were included three or four glucose standards and a blank. Each glucose standard contained 0.5 mg of glucose (i.e. 5 mL of the 0.1 mg/mL standard glucose solution) to which was added 10 mL of the anthrone reagent. The blank consisted of 5 mL of the 0.362 N sulfuric acid, to which also was added 10 mL of anthrone reagent. An accurate calibration curve of absorbance at 625 nm vs weight of glucose (in mg), was most simply prepared, by drawing a straight line through the origin and through the mean of the three or four measured absorbances at the single glucose weight of close to 0.5 mg. (Previous experiments had shown that plots of absorbance vs glucose weight did give a straight line which passed through the origin). Generally, a weight of 0.5 mg of glucose gave a mean absorbance of close to 1.666, with a standard deviation of between 0.002 and 0.010. In this case, therefore, the slope of the calibration curve was 3.333, and the weight of glucose in a 5 mL aliquot of diluted Klason filtrate was obtained simply by dividing the absorbance by this slope.

Absorbance Measurement

The absorbances of the blue-green reaction products, from the

anthrone reaction with the Klason filtrates and glucose standards, were measured at 625 nm against the blank. Before the room-temperature solutions were transferred to the spectrophotometer cells, the solutions were re-mixed on a vortex stirrer for 5s to re-incorporate any condensate from the walls. The re-mixed solutions were then transferred, in turn, to dry beakers and were poured into the sample cell. Removal and rinsing of the previous viscous and corrosive sample from the cell with the next solution, was easily accomplished without inverting the cell, by aspiration through a 2-mm teflon tube attached to a suction flask and extending down to the bottom of the cell.

Calculation of Hexosan Content

Provided the dilution is such that total absorbance is 0.5 or lower, and provided the pentosan content is no more than about 15% as great as that of the hexosan content, then no correction for pentosan interference is necessary. In this case, therefore, the weight of hexose (as glucose) can be read directly from the calibration curve of absorbance vs weight of pure glucose, or can be obtained simply by dividing the observed absorbance by the slope of the calibration curve (since the curve is a straight line passing through the origin).

If the pentosan content is much above 15% as great as that of the hexosan content, then a correction is desirable, for which some estimation of the amount of the pentosan is required. For this purpose the Tappi Standard pentosan analysis (T223 os-71) is suitable. If, however, the hexosan determination is being done

Table A-1 . Effect of Varying Glucose Concentration on the Xylose Contribution to Absorbance, at Constant Xylose Concentration (slopes and Y-intercepts of Straight lines of Fig. A-1

Constant Xylose concentration (mg/5mL)	Xylose absorbance[1] vs glucose concentration (mg/5mL)	
	Slope	Y-intercept
0.40	0.495	0.108
0.30	0.465	0.067
0.20	0.276	0.043
0.10	0.120	0.015
0.05	0.039	0.006

[1]Absorbance at 625 nm, produced when 10 mL of anthrone reagent is added to 5 mL of solution containing glucose and xylose, and mixture is held at 100°C for 4 minutes, minus the corresponding absorbance in the absence of the xylose

to compare with the glucan content obtained by HPLC, as was often the case in this work, then the xylan content from the HPLC is often suitable for this correction.

In Table A-1 are listed the slopes and Y intercepts of the five calibration curves of Fig. A-1. For each of these straight lines, the weight of xylose in the 5 mL of sugar solution used in the reaction with 10 mL of anthrone reagent was held constant, while the weight of glucose was varied. The equations of these lines are of the form:

$$A_{xyl} = m_{xyl} \cdot W_{glu} + b \qquad (1)$$

where: A_{xyl} is the absorbance contributed by the xylose

m_{xyl} is the slope

W_{glu} is the weight of glucose (in mg) in 5 mL of sugar solution

b is Y-intercept

These equations (Eq. 1) are valid, at constant xylose concentrations, over the ranges shown in Fig A-1. In this report, A_{xyl} is empirically defined as the amount by which the observed absorbance, of a xylose + glucose mixture, exceeds the absorbance which would have been obtained in the absence of the xylose, i.e.:

$$A_{total} = A_{glu} + A_{xyl} \qquad (2)$$

where: A_{total} is the observed absorbance obtained from a mixture of W_{glu} mg of glucose and W_{xyl} mg of

xylose, in
5 mL of sugar solution

A_{glu} is the absorbance which would have been obtained from W_{glu} mg of glucose alone in 5 mL of solution

A_{xyl} is the "xylose contribution to the absorbance" of the mixture of W_{glu} mg of glucose and W_{xyl} mg of xylose, in the 5 mL of sugar solution

The glucose calibration curve, over the range from 0 to 0.5 mg of glucose, was a straight line passing through the origin, with the equation:

$$A_{glu} = m_{glu} \cdot W_{glu} \qquad (3)$$

where A_{glu} is the absorbance obtained from W_{glu} mg of glucose, alone, in 5 mL of solution, when reacted with 10 mL of anthrone reagent

m_{glu} is the slope, which usually is very close to 3.33 but must be determined each day

Substitution of the value of A_{glu} from Eq. 3, in Eq. 2, and solving for A_{xyl}, gives:

$$A_{xyl} = A_{total} - m_{glu} \cdot W_{glu} \qquad (4)$$

Equating the right-hand sides of Eq. 1 and Eq. 4, and solving for W_{glu}, gives:

$$W_{glu} = \frac{A_{total} - b}{m_{glu} + m_{xyl}} \qquad (5)$$

The weight of glucose (W_{glu}) can therefore be calculated from Eq. 5, provided the slope (m_{xyl}) and intercept (b) of the appropriate xylose-contribution-to-absorbance curve of Fig. A-1 or Table A-1

are known. These values can be obtained accurately enough, by simple linear interpolation between the values listed in Table A-1 for the next higher and next lower weight of xylose.

For example, if the weight of xylose estimated to be present in the 5 mL of diluted Klason filtrate (as a result of either pentosan or HPLC analysis) were 0.15 mg, then the assumed xylose contribution curve would lie half-way between the curves for 0.20 and 0.10 mg of xylose (Table A-1). In this case, therefore,

$$m_{xyl} = 0.120 + 1/2\ (0.276 - 0.120) = 0.198$$

and $b = 0.015 + 1/2\ (0.043 - 0.015) = 0.029$

If, in this same case, the total measured absorbance were, for example, 0.559, and the slope of the glucose calibration curve were 3.33, then substitution in Eq. 5 would give:

$$W_{glu} = \frac{0.559 - 0.029}{3.33 + 0.198} = 0.150 \text{ mg}$$

The hexosan content, as glucan, in the original material subjected to the Klason lignin hydrolysis can then be calculated from:

$$\% \text{ hexosan} = \frac{W_{glu} \times \text{dilution factor} \times 0.9 \times 100\%}{5S} \qquad (6)$$

where: W_{glu} is the weight of glucose (in mg) from Eq. 5

S is dry equivalent weight (in grams) of hexosan-containing sample subjected to Klason lignin hydrolyses

dilution factor is the factor by which the 1 L of Klason filtrate was further diluted before the anthrone analysis (e.g. $\frac{100}{6}$ in the example given for aspenwood)

Effect of Mannose, Galactose and Uronic Acid

Mannose and galactose, like glucose, also produce hydroxymethylfurfural (HMF) which reacts with anthrone. If present in significant amounts they will contribute to the hexosan value obtained. Formation of HMF from mannose and galactose, however, is apparently slower than from glucose. Under the conditions of this modified anthrone procedure, mannose was found by experiment to have a glucose equivalent of 0.561. This means that mannose contributes only 56.1% as much as does an equal weight of glucose, to the measured absorbance. Mannose, like glucose, also enhances the absorption due to xylose. In this respect, mannose also "behaves" like 56.1% of its own weight of glucose.

The corresponding glucose equivalent of galactose was not determined in this work. However the value of 0.66 was reported in the book "Methods in Microbiology" edited by Norris, J.R. and Ribbons, D.W. [Vol. 5B, p. 271, 1971 Academic Press, London & N.Y.]. In the same table in this book, the glucose equivalent for mannose was listed as 0.55, which is in excellent agreement with the above value of 0.561. Furthermore, Koehler L.H. [Anal. Chem. 24, No. 10, 1576 (1952) "Differentiation of Carbohydrates by Anthrone Reaction, Rate and Color Intensity"] presented graphs which indicate that galactose had a glucose equivalent slightly greater than did mannose, in agreement with the above value of 0.66. This value was therefore accepted for galactose.

Glucuronic acid and glucuronolactone decarboxylate in the

concentrated hot sulfuric acid and are converted, like xylose, into furfural which reacts with the anthrone. The xylose equivalent of glucuronolactone was found by experiments to be 0.22, both in the absence of glucose, and when enhanced by glucose. This means that glucuronolactone "behaves" like 22% of its own weight of xylose in the absence of other sugars. Furthermore, when in the presence of glucose, the glucuronolactone contribution to the measured absorbance is also enhanced to the same extent as would that of 22% of its own weight of xylose. Interestingly, glucuronolactone also acts like 22% of its weight of xylose when submitted to the Tappi Standard Pentosan determination (which also involves a decarboxylation).

APPENDIX 2

Adjustment of Experimentally Determined Glucose Yield, Obtained on Enzymatic Hydrolysis of SHA-WI (80s/240°C) (Table 8), to the Calculated Yield to be Expected at the Lower Enzyme Loading (on a Per-Gram-of-Cellulose Basis) Used for SHA-WIA

The extreme right-hand column (Column 4) of Table 8 was obtained from Column 2, described below, using the curves of Fig. 20 of glucose yield vs enzyme loading. It is recognized that this adjustment, which permits comparison of glucose yields from SHA-WI with those from SHA-WIA on a per-gram-of-cellulose basis, is questionable at best, because Fig. 20 was obtained with an SO_2-catalyzed steaming, and a different enzyme preparation.

Table 7 shows that after an 80 s steam treatment at 240°C, the cellulose content (anthrone method) of the SHA-WI was 63.4%, whereas that of the SHA-WIA was much higher, at 86.6%. Equal enzyme loadings per gram of total substrate had been used. The enzyme loading, per gram of cellulose in the SHA-WIA, was therefore only $\frac{63.4}{86.6} = 0.73$ times as great as that per gram of cellulose in the SHA-WI. It was desired, therefore, to know what the glucose yield from SHA-WI would have been, if the enzyme loading had been only 0.73 as great.

Taking, for example, the glucose yield of 49% (shown in Column 2 of Table 8 for SHA-WI after 8 hours), and reading from the 6 h curve of Fig. 20, the corresponding enzyme loading of 11.0 FPU/g of total substrate can be read. Reducing this loading by the factor 0.73 (from the preceding paragraph) gives 11.0 x 0.73 =

8.1 FPU/g. Reading again from the 6 h curve, 8.1 FPU/g corresponds to a glucose yield of 38%. This calculated glucose yield is listed in Column 4 of Table 8, and can be seen to be the same as the 38% yield obtained experimentally for SHA-WIA (Column 3).

Similarly, using the 12 h curve, a 49% yield corresponds to an enzyme loading of 7.1 FPU/g. This loading, when reduced to 7.1 x 0.73 = 5.2 FPU/g, corresponds (with a very short extrapolation) to a glucose yield of 39%, i.e. very close to the 38% obtained with the 6 h curve.

The other three glucose yields (76, 85 and 86%) listed in Column 2 of Table 8, were similarly adjusted and gave the results included in Table A-2.

A-2.

Table A2. Calculation of Effect of Reducing Enzyme Loading to 0.73 of That Used in Experiment[1], on Expected Glucose Yield from SHA-WI (80s/240°C)

Hydrolysis time[2] (h)	Glucose yield by expt.[2] (%)	Corresponding enzyme loading from Fig. 20[3] (FPU/g)	(curve)	Reduced enzyme loading (Column 3 x 0.73)[4]	Calc'd yield at reduced enzyme loading[5] (%)
8	49	11.0	(6h)	8.1	38[6]
		7.8	(12h)	5.2	39
24	76	12.2	(12h)	8.9	59[6]
		8.3	(24h)	6.1	58
48	85	10.5	(24h)	7.7	72[6]
		7.7	(48h)	5.6	73
72	86	6.1	(72h)	4.5	76[6]

[1] For purpose of comparing with glucose yields from SHA-WIA at same FPU/g of cellulose

[2] Data from Table 8

[3] Enzyme loadings read from curves indicated, in Fig. 20, corresponding to the glucose yield listed in Column 2

[4] Factor 0.73 to reduce enzyme loading to that of SHA-WIA (per gram of cellulose)

[5] Yield read from same indicated curve of Fig. 20

[6] Values listed in Table 8

APPENDIX 3

Corrections Used in Table 15 to Adjust Concentrations of Glucose and Cellobiose

Aliquots of 3 mL each, were removed after intervals of 8, 24, 48, 72 and 96 hours, from the clear supernatant liquid of the enzymatic hydrolysate (original volume 200 mL). The removal of these aliquots, for analysis, reduced the volume of the remaining hydrolysis mixture, and also removed some of the glucose and cellobiose already formed. In order to adjust the observed concentrations, to what they should have been if no aliquots had been removed, the equations, listed below, were used. These equations assume that no enzyme components were removed in the aliquots withdrawn. This assumption is not entirely correct, specifically in the case of β-glucosidase. In most cases, however, the corrections were small.

$$C_{8cor} = C_8$$

$$C_{24cor} = C_{24} - \frac{v}{V}C_{24} + \frac{v}{V}C_8$$

$$C_{48cor} = C_{48} - \frac{2v}{V}C_{48} + \frac{v}{V}(C_8 + C_{24})$$

$$C_{72cor} = C_{72} - \frac{3v}{V}C_{72} + \frac{v}{V}(C_8 + C_{24} + C_{48})$$

$$C_{96cor} = C_{96} - \frac{4v}{V}C_{96} + \frac{v}{V}(C_8 + C_{24} + C_{48} + C_{72})$$

where: C_8, C_{24}, C_{48}, C_{72} and C_{96} are the observed concentrations after 8, 24, 48, 72 and 96 hours respt.

C_{8cor}, etc., are the corrected concentrations

v is volume of aliquot removed (3 mL in present case)

V is original volume of hydrolysis mixture (200 mL)